American Journal of Veterinary Research

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A Cytological Study of Costal Marrow. I. The Adult Horse

M. LOIS CALHOUN, D.V.M., Ph.D.

East Lansing, Michigan

IMPORTANT contributions have been made to the microscopic anatomy of bone marrow in man, monkey, and small laboratory animals. A search of the literature failed to reveal any similar readily available studies on farm animals. In view of the increasing importance of bone marrow as a diagnostic agent, and as a contribution to a field of histology relatively unexplored, a study of the bone marrow of the horse and cow* was undertaken.

LITERATURE REVIEW

The study of hematology has only recently come to the front in veterinary medicine. Wittmann[1] stressed its importance in the field of animal disease, veterinary surgery, and animal breeding. However, as early as 1906, Zuntz *et al.*[2] made histological studies on the bone marrow of dogs at sea level, and on animals acclimated to higher levels. Notable contributions on the subject of normal monkey bone marrow have been made by Suárez *et al.*[3] and Stasney and Higgins.[4] The science of hematology is indebted to Alexandrov,[5] Fairman and Whipple,[6] Stasney and Higgins,[7] Mulligan,[8,9] Van Loon and Clark,[10] Eberl,[11] and Bloom and Meyer[12] for studies on the bone marrow in the dog. In the first decade of this century, Külbs[13] worked on the development of bone marrow using the dog as an experimental animal. Bloom[14] compared blood and bone marrow in relation to pyometra in the dog.

A variety of studies has been carried out on rabbit bone marrow including the work of Lim, Sarkar, and Brown,[15] which entailed the establishing of the normal histological picture in a study of the effect of feeding thyroid, the work of Sabin and Doan[16] and Sabin *et al.*[17] on the bone marrow and blood in developing rabbits, the lymphocyte content by Yoffey and Parnell,[18] the distribution of bone marrow, bone, and bone ash,[19] and Jordan's[20] study of the giant cells of the rabbit and guinea pig bone marrow. Sundberg and Downey[21] compared the lymphoid cells of lymph nodes and bone marrow of rabbits and guinea pigs. Epstein and Tompkins[22] used the guinea pig as an experimental animal in a comparison of techniques for the differential counting of bone marrow cells. Mouriquand, Revol, and Edel[23] chose the femur as the site for a study of the myelogram in normal guinea pigs. Similar studies were made by Fairman and Corner,[24] Milman *et al.*,[25] Stasney and Higgins,[26] Kindred,[27,28] Plum,[29] and Endicott and Ott,[30] on the albino rat. A normal bone marrow study of white mice was made by Petri.[31] Hammon and Enders,[32,33] Lawrence *et al.*,[34] and Riser[35] studied the bone marrow in connection with panleukopenia of cats. Corradetti[36] studied the bone marrow of newborn cats in connection with some work on the blood and bone marrow of healthy human beings. Since this thesis was written, Sawitsky and Meyer[37] have published a paper on the bone marrow of normal cats.

Gütig[38] made a survey of the bone marrow in swine while studying the morphology of the blood of the species. Downey[39] used the adult guinea pig to determine the origin and development of both the eosinophil and the "hematogenous mast cell." Barbieri[40] studied the alteration of the bone marrow in association with hepatic distomoniasis in sheep. Ringoen[41] investigated the bone marrow of the sheep and pig to trace the origin of the eosinophil. Still other investigations serve to stress the growing importance of the bone marrow as an experimental organ and the need for knowledge of the normal. For example, Beilicke[42] observed the effect of iron on the blood and bone marrow of rabbits; Castrodale *et al.*[43] used dogs to study the comparative effects of estradiol and stilbestrol upon the blood, liver, and bone marrow; Menkin[44] studied the effect of the leukocytosis-promoting factor on the femoral bone marrow of dogs; Iantria[45] investigated the cytology of the erythroblast in the growing guinea pig embryo; Smith and Hastings[46] made a study of the megakaryocyte and blood platelet of the rat; Dougherty *et al.*[47] reported the changes in the myeloid and lymphoid tissues of estrogen-treated dogs; temperature variations between central and outlying bone marrow of the rabbit, pigeon, and albino rat were discussed by Huggins *et al.*;[48]

This paper is a condensation of a thesis submitted to the Graduate School of Iowa State College, Ames, in partial fulfillment of the requirements for the degree of doctor of philosophy.

* The study of the costal marrow of the cow will appear in the July, 1954, issue.

Huddleson and Munger[49] gave their attention to the phagocytic activity of bone marrow cells with the guinea pig as an experimental animal; Potter and Ward[50] investigated the development of the megakaryocyte in adult mice, and Nettleship[51] discussed the rabbit bone marrow changes produced by specific antibodies. Likewise Holderlin[52] studied the bone marrow and blood picture in the sensitized rabbit. The effects of sulfanilamide on the bone marrow of rats were presented by Higgins and Machella.[53] Damade and Leger[54] gave the cellular percentages in bone marrow following experimental anemia in the rabbit.

Little work has been done on the horse. Due to war conditions and failure to receive foreign journals, part of the work that has been done is not available. Ackerknecht[55] was probably the first to work on the horse. He spoke of the gross changes occurring with age and gave a brief description of the marrow cells. Varicak[56] studied the bone marrow of the trunk of domestic animals including horses. Hjärre and Berthelsen[57] presented detailed cellular counts of the bone marrow of 10 normal horses and compared them with a similar count in horses with infectious anemia. Tkachenko*[58] published a paper on the morphology of erythroblasts and myeloblasts in normal horse bone marrow. Hjärre[59] described the normal sternal punctate in the domestic animals including a comparison of the dog, pig, cow, and horse with man.

GROSS AND MICROSCOPIC ANATOMY OF BONE MARROW

The medullary cavity of the bones of the young of all species is filled with red marrow. As age increases, red bone marrow is gradually replaced by a labile, yellow, fatty marrow until, in the adult, red marrow is confined largely to the axial skeleton. According to Ackerknecht,[55] red marrow of the adult horse is confined to the proximal ends of the femur and humerus, the pelvic girdle, vertebrae, ribs, and sternum. Varicak[56] studied the bones of 100 horses macroscopically. While he determined no definite age for the transition from red to yellow marrow in horses, he rarely observed fatty areas in the axial skeleton of any horse under 8 years of age. In a study of the ribs of a 10-year-old horse, Varicak observed that active marrow was present for a distance of 20 cm. in the vertebral end, that the central part had both red and yellow marrow, while the sternal end was filled with fat. The fourteenth rib of the same animal had only a little fatty marrow in the ventral end. In a 30-year-old horse no fatty marrow was apparent macroscopically in the ribs. Ellenberger[60] reported gelatinous matter in older horses but Varicak,[56] working with healthy, active, aged horses, could not confirm this. Cowdry[61] had stated that gelatinous marrow should be expected in the aged human species. By studying the tibia, femur, rib, sternum, and vertebrae of man, Custer and Ahlfeldt[62] ascertained that the cellularity of red marrow decreased with advancing years. Specific gravity of bone marrow was found to be only slightly more than 1.0.[18]

Sisson and Grossman[63] attributed the blood supply of the long bones to the large medullary or nutrient artery which enters the nutrient foramen, extends through the canal in the compact bone, and ramifies in the marrow. A satellite vein follows the opposite course. Doan[64] found that periosteal vessels along the shaft, and some of the vessels near the extremities, furnished additional blood supply to the bone marrow. According to him, the thin walled venous sinusoids making up the vascular bed of the marrow are the most characteristic features of the gross circulation. Doan[64] and Doan *et al.*[65] contended that the vascular system is a closed system.

Osgood and Seaman[66] described the human marrow as the "largest, most widely dispersed, and least homogeneous organ in the body" with a volume one or two times that of the liver. Cowdry[67] stated that marrow makes up about 5 per cent of the total body weight. Mechanik[68] gave the weight of the bone marrow as 3.4 to 5.9 per cent of the body weight and estimated 5/9 Gm. of marrow to 1 Gm. of blood. Dietz[19] gave some interesting statistics on the relative weight of rabbit bone marrow: ⅓ the weight of the skeleton, 2.2 per cent of the total body weight, ⅔ the weight of the liver, and 50 times that of spleen.

According to Piney,[69] Ellenberger,[60] and Wolff,[70] there are no lymphatics in the marrow. Fischer[71] found lymph nodules in 38 out of 61 human cases investigated. Lymph nodules were considered by Mayer and Furuta[72] and Williams[73] to be a normal but variable constituent of human marrow. Ellenberger[60] credited man and cat marrow with lymph nodules. Wolff[70] did not find nodules with any regularity. Drinker and Yoffey[74] concluded that "follicular accumulations in the marrow do not seem to be true lymphoid nodules with cells showing active division."

Apparently, the nerve supply in man is confined to the walls of the blood vessels and no ganglion cells have been found.[70] Ackerknecht[55] found nerve bundles in the humerus of the horse.

MATERIAL AND METHODS

Obtaining the Samples.—The horses used in this investigation were a miscellaneous group of animals; 5 were brought to the Department of Veterinary Anatomy at Iowa State College, and 2 to the clinic at Michigan State College. All the horses were old but apparently free from disease as far as could be determined by general appearance and blood determinations.

The first problem was to determine a satisfactory place to secure a marrow sample. Since the horse has a heavy musculature covering the sternum, it seemed wise to try to find an area more accessible. Hjärre and Berthelsen[57] contended that

* Not available.

a sternal puncture was easily executed in the horse and they did not observe any complications from the procedure. The animal was confined in a prone position and an especially constructed trocar was driven into the middle or back sternebra by a "light" tapping with a hard rubber hammer. In any sternum that was examined by the author, no light tapping would succeed in penetrating the wall of the sternebrae. Also to have to confine such large animals in a prone position seemed a distinct disadvantage. According to Varicak,[56] the sternum of the horse was not suitable because of large muscles.

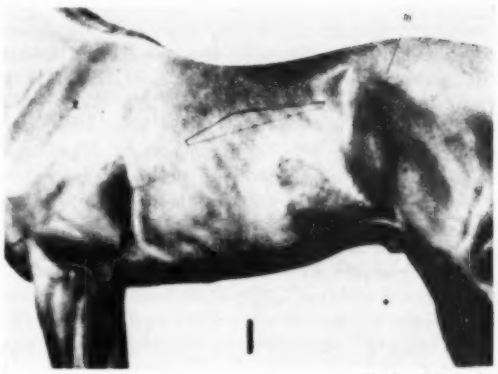
After several unsuccessful attempts to obtain marrow from the femur, ilium, and mandible of the horse, the author chose the ribs as the best site. Figure 1 shows the general area and figure 2 illustrates the fact that parts of the eighth to eighteenth ribs are relatively exposed in that region, being covered only by skin and fascia. It is well to go as high as possible and still avoid the latissimus dorsi (k) and serratus posticus (m) muscles because it seemed to be difficult to obtain sufficient marrow more ventrally. The animal may be confined in a stock or restrained against one side of a stall. Little or no resistance to the operation was ordinarily encountered. It is advisable to use a grooming brush on the back and side of the animal and to wipe the surrounding area, particularly above the prospective operative site, with a damp cloth to remove dust and particles which might later fall into the open wound.

The general area was palpated until the rib, which had the least amount of fascia covering it, was located. The chosen site was shaved, or the hair clipped closely, the area was washed with a soap solution, and iodine applied. A local anesthetic such as 2 per cent procaine hydrochloride was next injected into the skin, the underlying fascia, and finally into the periosteum, using about 10 cc. of solution. After a few minutes, a short incision was made in the skin, the fascia, and periosteum. A No. 487 Goodell-Pratt* hand drill, equipped with a straight shank $\frac{3}{32}$-in. jobbers' drill, was used to bore into the marrow cavity. A point midway between the anterior and posterior borders of the rib should be chosen for insertion of the drill to avoid missing the marrow cavity completely. Such an accident would entail the danger of penetrating the thoracic cavity. The drill "gives" when it hits the marrow, so it was not difficult to sense when the marrow was reached. The drill was removed from the rib and a cannula, with stylet of the same outside diameter as the drill, was inserted into the drill hole. A Jen-Sal needle trocar** was used. The stylet was removed and an airtight 10-cc. syringe attached to the cannula. One cubic centimeter or less of marrow was then drawn into the syringe.

The syringe was separated from the cannula and the marrow ejected into an oxalate tube to prevent coagulation (*see* later paragraph). The tube was held in a horizontal position and tapped to mix the marrow and oxalate. After obtaining the sample the cannula was withdrawn, iodine applied, and the incision left open. Healing took place rapidly and after the hair had grown out the site of the incision could not be determined. Varicak [56] found that trephining the ribs need not leave a scar. With the exception of the necessity for drilling through bone and the use of a cannula and stylet, the above procedure was patterned after that of Osgood and Brownlee [75] for sternal puncture in man.

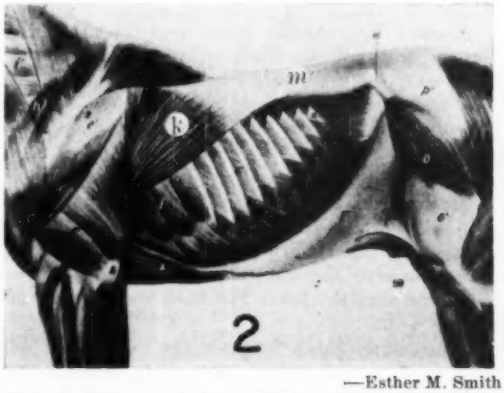
There has been some criticism of withdrawing more than 1 cc.[76] on the basis that the actual marrow withdrawn is too diluted with circulating blood. Jaffé [77] suggested 0.1 or 0.2 cc. According to Hjärre,[29] the sample should be more viscous than blood and should be greyish red.

It is possible to enter a large sinusoid and withdraw material that differs little from peripheral



—Esther M. Smith

Fig. 1—The general area (the ribs) of the horse from which marrow was obtained for this study.



—Esther M. Smith

Fig. 2—Parts of the eighth to the eighteenth ribs in the horse are relatively exposed, being covered only by skin and fascia.

* Goodall-Pratt Company, Greenfield, Mass.

** J.S. 39,121 in the 1953 catalogue, Jen-Sal Laboratories, Kansas City, Mo.

blood.[59, 78] Should this occur, the procedure should be repeated at a different level or on a different rib. Slides were taken to the operative site in event fresh smears were desired.

The state of the marrow can be determined only after analyzing the blood picture.[79] Manaugh [80] suggested that correlation of the marrow picture with that of the peripheral blood might eventually result in the ability to read the marrow hemogram by the blood findings alone.

Blood samples were obtained prior to procuring the marrow as the latter procedure might excite the animal enough to alter the blood picture. The usual aseptic venipuncture technique for large animals described by Conner [81] was followed.

The samples were taken to the laboratory and blood and bone marrow smears made immediately. Total red and white counts were done on the blood and the amount of hemoglobin determined. Osgood's [82] technique was followed. Total red and nucleated cell counts on the marrow samples were purposely omitted because there was such a large variation due to dilution with circulating blood that the data seemed to have little or no value. Nordensen,[83] and Kandel and Leroy,[84] working with human bone marrow, also found this to be true.

The above technique was designed for the living animal and gives the optimum normal cytological picture. However, there may be occasions when postmortem marrow examination would be necessary. Wintrobe [85] felt the sample should be secured within two hours after death to get good preparations as autolysis occurs soon. Rohr and Hafter [86] made a special study of postmortem changes in human bone marrow. They concluded that death had little effect on the myeloid elements, but the erythroblast matured with a subsequent change from polychromatic to oxyphilic forms and extrusion of the nuclei. They also observed a decrease in the number of neutrophils beginning a few minutes postmortem. The nuclei of the remaining neutrophils swelled and became vacuolated after two hours.

Oxalate Tubes.—The blood was collected in ordinary test tubes. Dunham fermentation tubes were used for the marrow because they were short and had a relatively small bore; consequently, the marrow and oxalate could be mixed more thoroughly.

The oxalate tubes were prepared by evaporating to dryness 0.1 cc. of a 2 per cent potassium oxalate solution for each cubic centimeter of blood or marrow (2 mg. of dry potassium oxalate per cc.). The requisite amount of oxalate solution was measured into the tubes and they were placed in a drying oven.

Maurer and Jones [87] evaporated the oxalate solution at a temperature below 80 C., since higher temperatures converted some of the potassium oxalate to carbonate and consequently coagulation was not inhibited. Too much oxalate tended to distort the normal cell structure. The cells appeared shrunken and the staining was too intense. Osgood, Haskins, and Trotman [88] recommended oxalate blood because of greater convenience and believed its use resulted in a higher degree of accuracy; since the sample was larger, more time could be taken and duplicate estimations could be run. They suggested a 24-hour time limit for hemoglobin readings and total red and white blood cell counts, and a one-hour limit for the smear for a differential. In some investigations at the University of Minnesota, Kernkamp [89] concluded that if oxalated blood samples were examined within sixty to ninety minutes after obtaining the blood, few changes resulted. Maurer and Jones [87] agreed with this view.

Staining and Counting Technique.—The slides were soaked in sulphuric acid-potassium dichromate cleaning solution for twenty-four hours, rinsed in running tap water for another twenty-four hours, rinsed in distilled water, and stored in 70 per cent alcohol. They were dried as needed. A 24-gauge bacteriology platinum wire loop was used to remove the blood and marrow from the tubes in making the smears.

Wright's staining technique, as outlined by Osgood and Ashworth,[90] was followed. Wright's dry stain, 0.1 Gm., was mixed with 20 cc. of acetone-free methyl alcohol and left overnight before use. A buffer solution with a pH of 6.4 was prepared by dissolving 6.63 Gm. of monopotassium phosphate and 2.56 Gm. of anhydrous disodium phosphate in a liter of distilled water and adding 1 cc. of chloroform. Six to 12 drops of the dye, depending on the size of the smear, were left on the slide for two minutes, then diluted with an equal number of drops of buffer, and left seven minutes longer. With the slide in a horizontal position, the staining solution was rinsed off, the slide washed for thirty seconds with a brisk stream of running tap water, then air-dried. No cover slip was applied; instead, the smear was covered with a thin film of immersion oil at the time of examination.

In this study, the filled counting chambers were examined with the low power objective for general uniformity of distribution, bubbles, or any foreign material. If the initial survey revealed no inequality of cell dispersion, bubbles, or other artifacts, the counts were made. Differences exceeding the "standard limits" were not discredited. Both sides of the chamber were counted and totals were determined from those figures.

Hemoglobin readings were made on a Dare hemoglobinometer. Although this instrument would not be precise enough for research specifically on hemoglobin, it was deemed sufficiently accurate for use here.

Plum [91] was of the opinion that the unequal distribution of the cells in the blood smear played the predominant role in the error of individual counts. MacGregor *et al.*[92] claimed that the "battlement edge" count gave the greatest accuracy. This consisted of starting on the edge of the smear, going a millimeter in from the edge, the same dis-

tance across, another millimeter back to the edge, and an equal distance horizontally along the edge, continuing this pattern in the same direction until 300 cells had been counted.

Hay [93] recommended counting 300 or 400 white blood cells to reduce the error of random sampling. He found further that the choice of area in which to count the cells did not play as important a part as thought by earlier investigators.

With the low-power magnification, the author chose a field in which there was an even distribution of nucleated cells, the red cells did not overlap, and staining was sharp. The edge of the smear was avoided. In the blood smears, 300 cells were counted at random by going back and forth across the smear. All differential counts were made with a magnification of 855 times. A Spencer lamp, model 370, was the source of light. A 100-watt bulb was customarily used but for cytological details a 200- or even a 300-watt bulb will bring out special structures.

Thoma pipettes were used throughout for diluting the blood for total counts. The Neubauer bright line counting chamber was employed. Separate counting chambers were used for the red and white counts. This avoided any possibility of carrying over any of the acetic acid used in the white count to that of the red count.

Standard textbooks on hematological techniques call for a variation of less than 18 to 20 cells between the five groups of squares for the red count, and variation not to exceed eight or ten cells between squares in the white count. This would be within limits of 180,000 to 200,000 and 160 to 200 cells, respectively, for total counts. Windle *et al.*[94] accepted an agreement of two counts within limits of 100,000 for the red blood cells and 250 for white blood cells. Berkson *et al.*[95] and Magath *et al.*[96] showed that such stringent limits are not statistically probable. To the contrary, they found that greater differences than those would be normally expected in 50 to more than 90 per cent of the counts. Ohlson [97] made a study of sampling methods in connection with erythrocyte determinations. From data obtained with hemocytometers that presented a uniform distribution of cells and no bubbles, the following conclusions were drawn: (1) Significant differences occurred between subjects; (2) differences bordering on significance might occur between pipettes; (3) with the trained technician there was no statistically significant difference between successive counts on the same sample even though differences exceeding 20 cells existed between the five groups of squares.

In the differential cell count of the marrow, 500 cells were enumerated. Stasney and Higgins,[98, 98] Osgood,[99] Kingery *et al.*,[100] Pitts and Packham,[101] and Bloom and Meyer [12] enumerated 500 cells. Wintrobe [85] stated that 500 to 1,000 should be counted. Davidson *et al.*[102] suggested observing 400 or 500 cells, Hjärre [59] counted 800 cells, and Wilson [103] and Thomson [104] counted 1,000 cells. Reich *et al.*[105] did not do differential counts "owing to the inaccuracy of the method." In a subsequent personal communication, Dr. Reich said that the inaccuracies they found might not exist in this work.

According to Cowdry,[67] a bone marrow smear will contain endothelial cells from the capillaries, some reticular cells, and connective tissue fibers in addition to the developing blood cells. Cells are frequently injured and the cytoplasm may have been torn and the nucleus drawn out in strands. Other cells appear to be dying. These injured and dying cells have been given various names such as degenerate, smudge, smear, or basket cells. Since Hynes [106] considered these cells to be "largely fortuitous and merely distort the picture if they are included in the differential count" and since Bloom [107] thought them artifacts and not necessary to include, it was decided to omit them from the differential count.

Bone Marrow Studies

Terminology.—In 1912, Ackerknecht [65] remarked that no unity existed in the morphological and histogenetic classification of marrow cells. In 1944, Osgood and Seaman [66] were still pleading for "an approved 'Standard Nomenclature for Hematology'."

Most authors have adhered to a generally uniform set of terms in describing bone marrow cells. Unfortunately, the same term did not always apply to the same type of cell. After analyzing the various nomenclatures used, the following relatively simple, useful terminology was decided upon.

Stem cell—all the immature cells which could not be classified into any given series.

Erythroblast—all cells in the red blood cell series from the youngest that could be identified with that series to the normoblast.

Normoblast—those in the red blood cell series that contained hemoglobin in comparable amounts to the adult red blood cells as indicated by similar staining properties and containing nuclei.

Promyelocyte—young cells with nonspecific azurophilic granules.

Eosinophilic and neutrophilic myelocytes—cells in these respective series included the metamyelocytes, juvenile cells and stab, rod or band forms of other authors.

Eosinophil and neutrophil—the adult cells of these granulocyte series.

Basophil—there were so few basophils that both developing cells and adults of that series were grouped together.

Lymphocyte—any developing cells in this series were included.

Monocyte—all promonocytes and distinguishable monoblasts were added here.

Plasma cell—if any developing plasma cells were observed, this heading included them.

Since this thesis was written, a committee for clarification of the nomenclature of cells and diseases of the blood and blood-forming organs has been organized under the leadership of Dr. Edwin

TABLE 1—Percentage of Distribution of the Marrow Cells from the Ribs of Seven Horses

Cell Types	6943w F	6843b F	6843w F	7943b F	6943s M-c	8344 M-c	92944 F	Range	Mean
Stem Cell	0.4	0.4	1.6	3.4	2.0	2.4	1.0	0.4 to 3.4	1.6
Erythroblast	19.4	8.0	14.0	32.0	23.6	31.4	18.2	8.0 to 32.0	20.94
Normoblast	24.2	12.0	5.0	15.6	15.2	13.6	10.4	5.0 to 24.2	13.71
Total erythroid cells (E)	43.6	20.0	19.0	47.6	38.8	45.0	28.6	19.0 to 47.6	34.66
Promyelocyte	0.0	0.6	5.0	1.8	1.6	3.2	0.6	0.0 to 5.0	1.83
Neutrophilic myelocyte	26.2	47.8	56.0	26.6	31.6	37.8	40.4	26.2 to 56.0	38.06
Neutrophil	20.2	16.6	9.0	12.6	15.4	1.8	17.6	1.8 to 20.2	13.31
Eosinophilic myelocyte	0.8	3.4	0.4	2.6	3.6	2.6	3.0	0.4 to 3.6	2.34
Eosinophil	0.4	0.2	1.0	1.0	0.2	0.2	1.2	0.2 to 1.2	0.60
Basophils (all)	0.0	1.0	0.2	0.4	0.8	1.0	0.8	0.0 to 1.0	0.60
Total myeloid cells (M)	47.6	69.6	71.6	45.0	53.2	46.6	63.6	45.0 to 71.6	56.74
Monocyte	2.0	4.4	4.8	1.2	1.6	1.8	1.4	1.2 to 4.8	2.46
Plasma cell	0.8	0.6	0.8	0.8	0.6	0.8	0.0	0.0 to 0.8	0.63
Lymphocyte	5.6	5.0	2.2	2.0	3.8	3.4	5.4	2.0 to 5.6	3.91
Megakaryocytes in 300 sq. mm	0.0	0.0	8.0	0.0	3.0	1.0	0.0	0.0 to 8.0	1.71
Mitoses	2.0	0.0	0.0	2.0	8.0	1.0	6.0	0.0 to 8.0	2.71
Myeloid-erythroid ratio	1.09	3.48	3.76	0.94	1.37	1.04	2.22	0.94 to 3.76	1.64

M-c, male castrate; F, female.

E. Osgood, chairman, Portland, Ore. Their first report [108] has been published.

Myelograms.—Table 1 indicates the individual myelograms for all the experimental animals in this investigation.

Table 2 contains a summary of this study and a comparison with similar investigations carried on elsewhere.

In this study, the slides were examined in the following manner. A low-power preview was made to get an idea of distribution, staining, and anything unusual. A representative field was chosen for the differential counts, measurements, color comparisons, and cytological details and was explored under oil immersion.

The two main sources of reference used in identifying and describing the cells were "Atlas of Hematology" by Osgood and Ashworth,[80] and the "Munsell Book of Color."[109] "Colour Terminology in Biology" by Dade [110] was occasionally referred to.

For the purpose of specificity, color comparisons were made with the Munsell color book and the approximate color or range of colors was determined. Comparing transmitted light with reflected light presented some incongruities. The most predominating nuclear color was chosen for comparison. The nuclear color of all cells may be approximately matched in the 5.0 red-purple color chart. The darkest nucleus with a value of 2 and a chroma of 4 (2/4) was found in the normoblast. The pale staining which was frequently found in the monocyte nucleus may be designated 7/4. Other cell nuclei matched colors lying between these two extremes depending on the stage of development and the intensity of staining.

Like that of the nucleus, the cytoplasmic color varied with the degree of staining but in addition there was considerably more variation between cells. Frequently, the cytoplasm was so pale that it might be des-

TABLE 2—Comparison of Horse Data with Other Myelograms

Hjärre and Berthelsen [87]	10 horses	Hjärre [89]	Author	Range from table 1	Mean from table 1
Myeloblast	1.5 to 2.5	1.5 to 3.0	Stem cell	0.4 to 3.4	1.6
Promegaloblast-like cells	1.0 to 2.0	.....			
Pronormoblast	1.5 to 3.0	2.5 to 5.5	Erythroblasts	8.0 to 32.0	20.94
Basophilic normoblast	4.5 to 9.0	4.5 to 9.0	Normoblasts	5.0 to 24.2	13.71
Hb. containing normoblasts	40.0 to 60.0	40.0 to 60.0	Total erythroid cells	19.0 to 47.6	34.66
Promyelocyte	1.0 to 2.5	1.2 to 5.0	Promyelocyte	0.0 to 5.0	1.83
Myelocyte	2.0 to 5.0	2.0 to 6.7			
Metamyelocyte	5.0 to 15.0	5.0 to 15.7	Neutrophilic myelocyte	26.2 to 56.0	38.06
Stab cells	5.5 to 11.0	5.0 to 10.7	Neutrophil	1.8 to 20.2	13.31
Segmented neutrophils	6.5 to 14.0	6.3 to 16.0	Eosinophilic myelocyte	0.4 to 3.6	2.34
Eosinophil	0.5 to 1.5	.....	Eosinophil	0.2 to 1.2	0.60
Basophil	0.0 to 0.5	.....	All basophils	0.0 to 1.0	0.60
			Total myeloid cells	45.0 to 71.6	56.74
Monocyte	0.0 to 0.5	0.0 to 0.5	Monocytes	1.2 to 4.8	2.46
Plasma cell	0.1 to 1.0	.....	Plasma cell	0.0 to 0.8	0.63
Lymphocyte	2.0 to 6.0	.....	Lymphocyte	2.0 to 5.6	3.91
Myeloid-erythroid (M/E) ratio	.....	0.5	M/E	0.94 to 3.76	1.64 (T.1)

ignated "colorless." The pale blue of the monocyte and a color in the erythroblast series compared favorably with 8/2 in the 5.0 yellow-red plate. The color of the extranuclear protoplasm of other cells ranged from 8/2 to 5/8 inclusive on the 5.0 purple-blue plate.

The various shades of the eosinophil granules may be designated by 5.0 red 8/4, 10.0 red-purple-red 8/6, 5.0 red-purple 7/2, 7/4, 6/2 to 6/8, 5/6 and 5/8. The basophilic granules which were intermingled with the eosinophilic granules were much the same color as those of the basophil itself.

The basophil granules matched colors under the classification 5.0 red-purple 2/4, 2/6, and 3/6 in the Munsell color book.

The azurophil granules of the lymphocyte, monocyte, and promyelocyte were about the colors 4/10, 4/12, and 5/10 in the 5.0 red-purple plate.

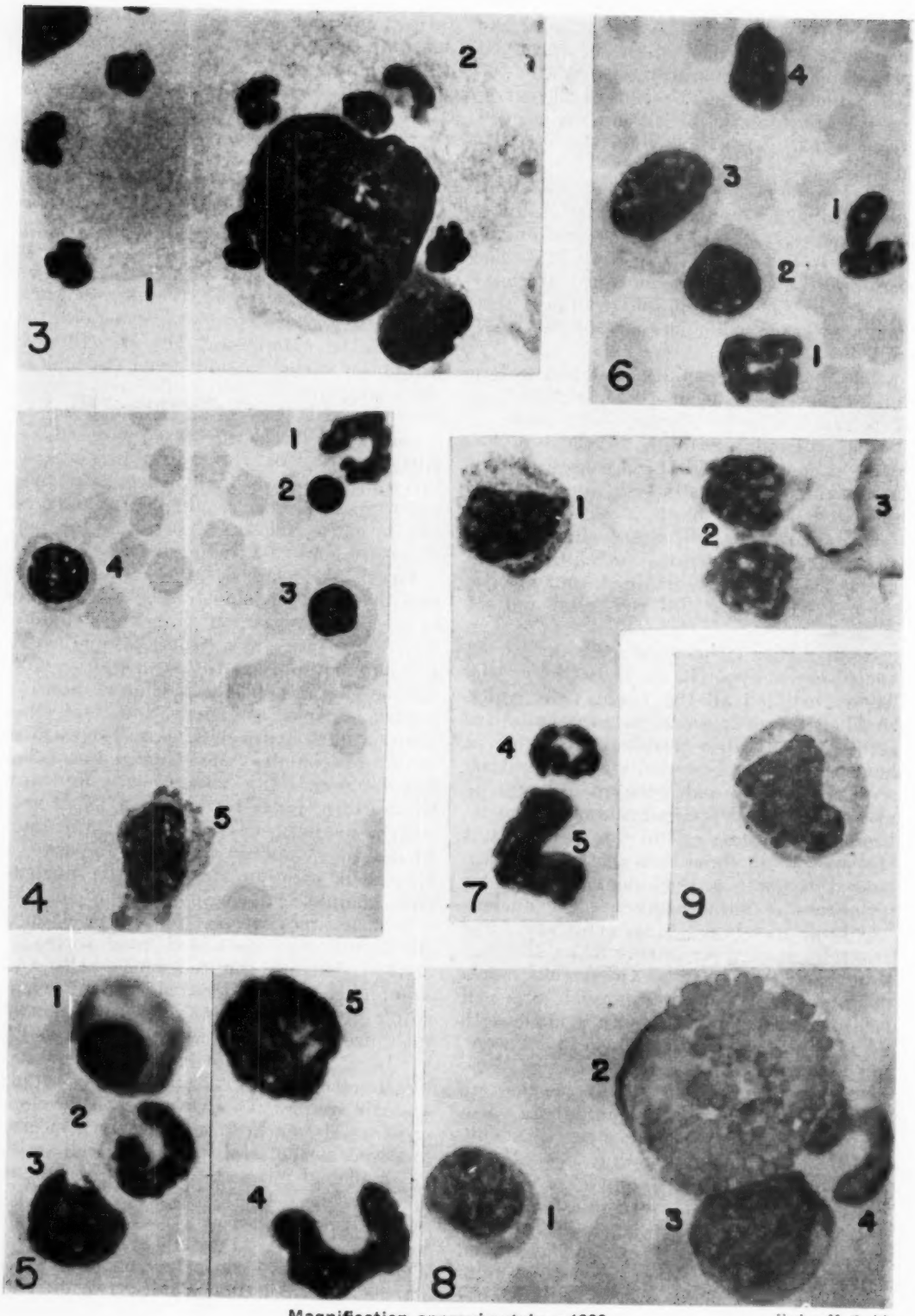
The smallness of the neutrophil granules made color comparisons difficult but, as nearly as could be ascertained, they are the color of 8/4 in the 10R red-yellow-red set of hues.

Stem Cell. — The stem cells measured varied in size from 12 by 14 to 26 by 30 μ. These included all the young cells which could not be classified in any definitive series. The reddish purple nucleus had a homogeneous finely reticulated karyoplasm with two or more pale blue nucleoli. Bloom and Meyer [12] did not find a nucleolar membrane in the stem cell of dog marrow but the nucleoli in these cells in the horse appeared to have a very definite nucleolar membrane in most instances. The nucleus was large in relation to its cytoplasm. For example, one cell measuring 22 by 22 μ had a nucleus 14.5 by 16 μ. Other cells had a narrower rim of cytoplasm such as a cell measuring 13.5 by 17 μ with a nucleus 10 by 15 μ. The cytoplasm, which had a varying color range from a pale blue to a greyish sky-blue, often presented a mottled appearance and frequently contained a vacuole or two. Some of the stem cells contained a few azure-staining granules in the cytoplasm (fig. 4, 5, and 7).

Erythroblast and Normoblast.—Davidson, Davis, and Innes [102] have aptly defined the erythroblast as "any nucleated cell capable of differentiation toward an erythrocyte" and divided such cells into four groups including the stem cells of that series and the orthrochromatic erythroblast or normoblast. The erythroblasts here include only two groups, the basophilic erythroblasts and those beginning to take up hemoglobin (the polychromatic erythroblasts of some authors). Those measured varied in size from 7 by 7 to 15 by 20 μ. One in the process of mitosis was 15 by 23 μ. The nucleus varied from the homogeneous reddish purple, nucleoli-containing nucleus of the most primitive erythroblast to the chromatin-clumped dark purple nucleus of the late erythroblast just prior to complete hemoglobinization of the cytoplasm. The structureless cytoplasm varied in color from the blue of the more primitive cells to the steel grey or even greenish grey of the stage near the normoblast. The normoblast presented size variations of 5 by 7 or 6 by 6 to 9 by 9 μ. One in mitosis was 12 by 12 μ. The pyknotic nucleus was so purple it appeared almost black and was so dense no internal structure could be distinguished.

Israels [111, 112] has described the maturation of the erythroblast. The cell shrinks to one half its original size; the cytoplasmic color progresses from basophilic, to polychromatophilic, to eosinophilic with the increase in hemoglobin content, and the nucleus shrinks, condenses, and finally becomes a dark featureless mass. The nuclear structure changes and hemoglobinization are not necessarily synchronized into any set pattern for occasionally a small cell with a pyknotic nucleus may retain quite basophilic cytoplasm while the opposite, a large cell with an erythroblastic nucleus and complete hemoglobinization of the cytoplasm, may occur. Israels [111] claimed the latter was associated with some increased demand for red blood cells and should not be considered normal. For example in horse No. 6843b, cells with hemoglobinized cytoplasm were as large as 13 by 14 μ. This animal had the lowest red blood cell count (4,905,000) of any of the animals studied. This variation in development need not be a matter for concern, however, as the total for the red cell series is a sufficient index to the state of the bone marrow (fig. 3 to 8).

Promyelocyte.—The promyelocytes varied in size from 15 by 16 to 21 by 22 μ. This cell had a spherical or ovoid reddish purple nucleus with usually two to five pale blue nucleoli. The light blue cyto-



Magnification approximately x 1300.

—Esther M. Smith

plasm contained small, fairly evenly distributed azurophilic granules. Sometimes a rim of deeper blue cytoplasm was present (fig. 6 and 7).

Neutrophilic Myelocyte.—The cells grouped in this series included several developmental stages with morphological variations but all had about the same staining properties. The size ranged from 10 by 13 to 16 by 18 μ. The reddish purple nucleus varied in form from the spherical "finely chromatined" nucleus of the youngest cell in the series through successive stages of oval, kidney bean, and u-shapes to the darker staining, "chromatin-clumped" nucleus beginning to show constrictions but not segmented into lobes. These nuclear changes correspond to the myelocyte, metamyelocyte or juvenile, and staff cells of other authors. The cytoplasm varied from colorless to pale blue with fine neutrophilic granules. The early stages often retained a few larger azurophilic granules (fig. 4 to 8).

Eosinophilic Myelocyte.—These cells pass through the same developmental stages as the neutrophil. They are easily identified by the eosinophilic granules which vary from 1 to 3 μ. From 30 to as many as 125 granules were seen in a single cell.

Hirschfeld [113] counted from 20 to 40 granules. He stated that the largest ones might reach the size of the goat erythrocyte. The largest cell measured was 23 by 33 μ. In the young cells, there was considerable variation in color of the granules, some azurophilic, others taking on a blue-grey shade. Most of the granules were spherical but a few oval ones were observed in the horse eosinophil. Hirschfeld [113] also observed these elliptical granules. According to Downey [39] the eosinophilic granules are endogenous, being differentiated from the basophilic protoplasm. He found that they changed from small basophilic granules to even larger eosinophilic ones than were to be found in the mature eosinophil.

The cytoplasm appeared grey-blue when not obscured by the granules. The reddish purple nucleus changed from spherical to ovoid to u-shaped, becoming lobed in the adult cells. One or more nucleoli were observed in the most immature cells of this series (fig. 8).

Basophils.—The largest immature basophil measured was 18 by 21 μ and the smallest adult cell in this series was 11 by 11 μ. The nucleus when discernible was a reddish purple color but it was frequently masked by the basophilic granules which were a deeper shade than the nucleus. The spherical granules varied in size, the largest being about a micron in diameter. The cytoplasm was a pale blue. According to Hirschfeld,[113] the mast cell (basophil) of the horse was large and displayed an abundance of granules such as did not occur in any other animal. He thought the granules were needle shaped (nadel-formig). Neither particularly large basophils or needle-shaped granules were observed by this author. Staining variations of the granules ranged from a muddy reddish color to a very dark purple with a brownish cast. Figure 5 includes a basophil.

Plasma Cell.—The plasma cell of the horse was easily distinguished from other cells but its differential characteristics are difficult to describe. The blue cytoplasm, just a bit brighter than that of the erythroblast, and the usually eccentrically placed nucleus, with its perinuclear clear area, all combined to set it apart from similarly stained cells in the lymphocytic and red blood cell series. The largest plasma cell measured was 14 by 19 μ with an 8 by 8 nucleus. A smaller cell (11 by 11) had a nucleus 7 by 8 μ. The chromatin network was heavy and coarse textured in the older cells but of a finer structure and less dense in more immature cells. Michels [114] has

Legends for Illustrations on Opposite Page

Fig. 3—Megakaryocyte showing platelets budding off at 2; 1, cell border.

Fig. 4—1, Neutrophilic band cells; 2, normoblast nucleus; 3, normoblast; 4, erythroblast (late); 5, stem cell.

Fig. 5—1, Plasma cell; 2, neutrophilic band cell; 3, stem cell; 4, neutrophilic metamyelocyte; 5, basophil.

Fig. 6—1, Neutrophilic band cells; 2, erythroblast (late); 3, promyelocyte; 4, neutrophilic myelocyte.

Fig. 7—1, Promyelocyte; 2, stem cells; 3, degenerating cell; 4, neutrophilic band cell beginning to segment; 5, neutrophilic metamyelocyte.

Fig. 8—1, Erythroblast; 2, eosinophilic myelocyte; 3, erythroblast (young); 4, neutrophilic band cell.

Fig. 9—Promonocyte.

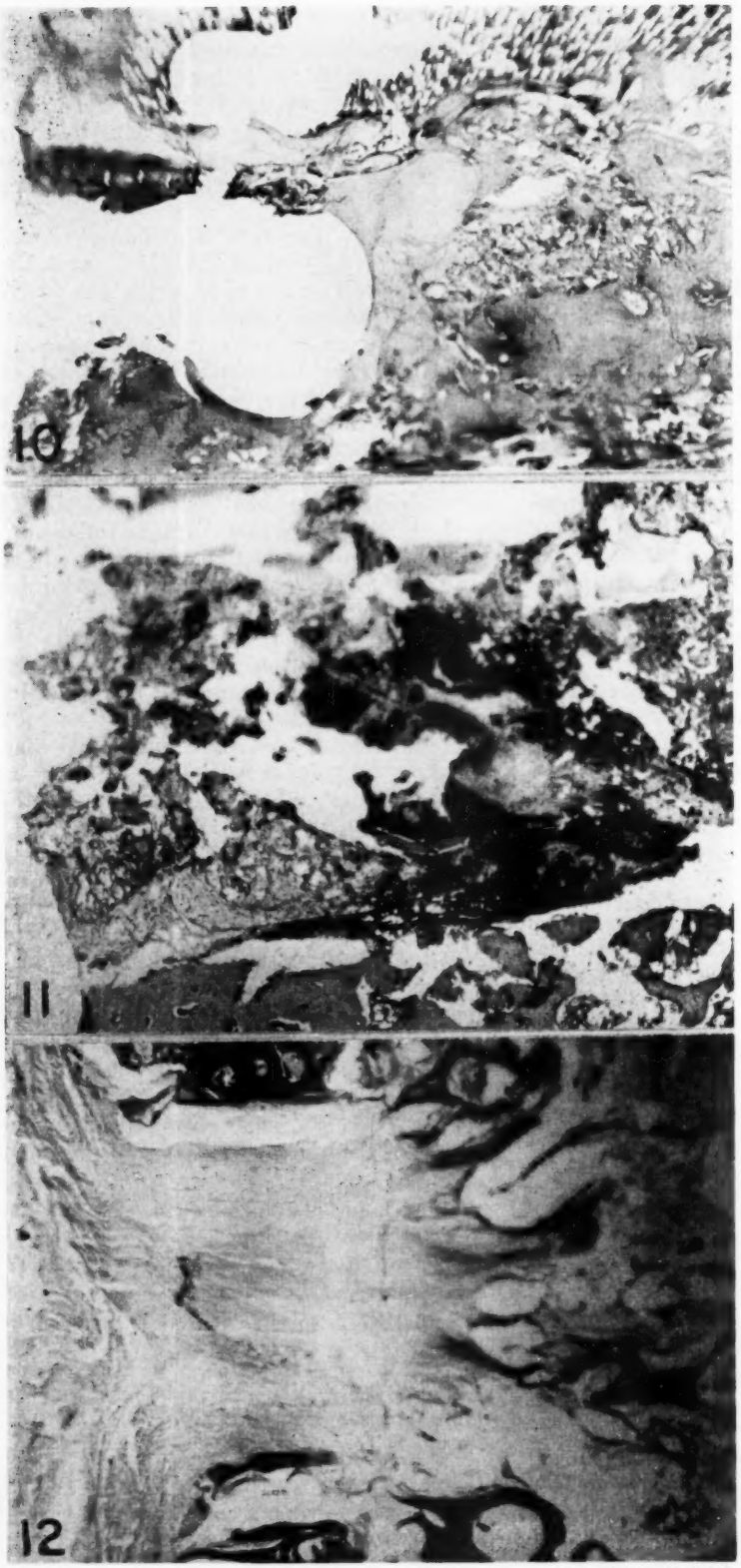


Fig. 10 — Rib drilled two hours before the animal was killed, showing fibrin in the blood clot. x 30.

Fig. 11 — Rib drilled two days before the animal died. Numbers of fibroblasts were greatly increased. x 30.

Fig. 12—Rib drilled fifteen days before the animal was killed; provisional callus formed. x 30.

—Esther M. Smith

written a review on the plasma cell including morphogenesis (fig. 5).

Megakaryocyte. — Ackerknecht [55] described two types of megakaryocytes in the horse, one a multinucleated cell, the other a single nucleated cell having somewhat different staining properties. He found transitional stages between the two. He suggested that the multinucleated cells might be a regressive product of metamorphosis. Kingsley [115] made a study of the development of the megakaryocyte in pig embryos. According to him, the megakaryocytoblast is a small cell (8 μ) and can be distinguished from the hemocytoblast by specific cytoplasmic granules. The cell increases in size, the spherical nucleus becomes oval, then horseshoe shaped, and finally bell-shaped. Disappearance of the nucleoli and vesiculation of the nucleus accompany these changes. Subsequent development includes increase in size and amount of the cytoplasm and nucleus. It may have been these different stages in development that Ackerknecht [55] observed. Limarzi and Schleicher [116] included the steps in the maturation of the megakaryocyte in a study of thrombopenic purpura. They grouped them into young, adult, and degenerated types. Levy [117] concluded that they are abnormal cells, an outcome of malformed cell divisions and no special function should be given to them. Whatever their role, that of platelet formation or merely functionless developmental abnormalities, a description follows.

The megakaryocyte is the largest cell in marrow. Its size varied from 15 by 21 to 85 by 100 μ. The cells were spherical or ovoid. Some of them had large pseudopodia. Very few were encountered in the horse and many of those did not seem to have any cytoplasm. According to Limarzi and Schleicher,[116] platelets are formed by either a detachment of portions of the cytoplasm or by cytolysis. Schenker [118] favored platelet formation through disintegration of mature megakaryocyte plasma and found evidence to support the theory that the platelets are of nuclear origin (fig. 3).

Mitosis.—Table 1 indicates the number of mitotic figures encountered in identifying and enumerating 500 cells. They were chiefly in the red blood cell series but examples of all types of cells were observed in mitosis.

According to Osgood,[119] all the "blast" and "pro" cells divide by mitotic division and the more mature erythroblasts, plasmacytes, and lymphocytes divide amitotically. Japa [120] studied cell division in human marrow and found the normal number to be 15 per 100 nucleated cells distributed as follows: 40 per cent prophase, 45 per cent metaphase, 10 per cent anaphase, and 5 per cent telophase.

Myeloid-Erythroid Ratio.—The myeloid-erythroid ratio, the ratio of developing red blood cells to those in the white blood cell series, is an important index of the activity of the marrow. Should a systemic demand for an increase in one or the other of these series be reflected in the marrow, the usual ratio would be upset. This ratio was determined by adding the percentages of erythroblasts and normoblasts and comparing the figure with a similar sum of the developing granulocytes. These are indicated in table 1 by "total myeloid cells" and "total erythroid cells." The myeloid-erythroid ratio (M/E), given in the same table, varies from 0.94 to 3.76 in the horse. The mean M/E ratio for the horses in this study was 56.74/34.66 or 1.64.

Bone-Healing Process.—Figures 10 to 14 show the healing process that follows the use of the drill in procuring marrow from the rib of the horse. The surgical procedure of obtaining marrow was carried out at the following intervals before the animal was killed:

Fifty-one days, 44 days, 37 days, 30 days, 23 days, 15 days, 9 days, 4 days, 2 days, 6½ hours, and 2 hours. At necropsy, the portion of the ribs including the site of the drill hole was obtained. These were subsequently decalcified, embedded in paraffin, and histological sections prepared. Figures 10 and 11 show consecutive stages in the early healing process. High-power magnification showed an increasing number of fibroblasts with the lengthened time interval between the operation and killing of the animal. Figure 12 shows the drilled area filled with a loose fibrous connective tissue. Figures 13 and 14 show bone filling the drill site and in the latter healing is practically completed. The bony lamellae appear to extend parallel to the direction of the drilled hole. Figure 15 shows a longitudinal section of a rib which had not been drilled. By reviewing these slides, it appears that the healing proceeds along the same pattern as an ordinary fracture. Nothing but intramembranous bone formation was observed.

DISCUSSION

Hematologists are not agreed on the value of aspiration and bone marrow smear methods. Doan and Zerfas [121] suggested conservatism in drawing deductions because of the limitations of the technique, the fallacy of drawing conclusions from counting so few of the millions of cells present, and the debated question of identification and classification. Custer [122] found the cellular state of marrow to vary

Fig. 13 — Rib drilled thirty-seven days before killing the animal; drill hole filling with new bone. x 20.

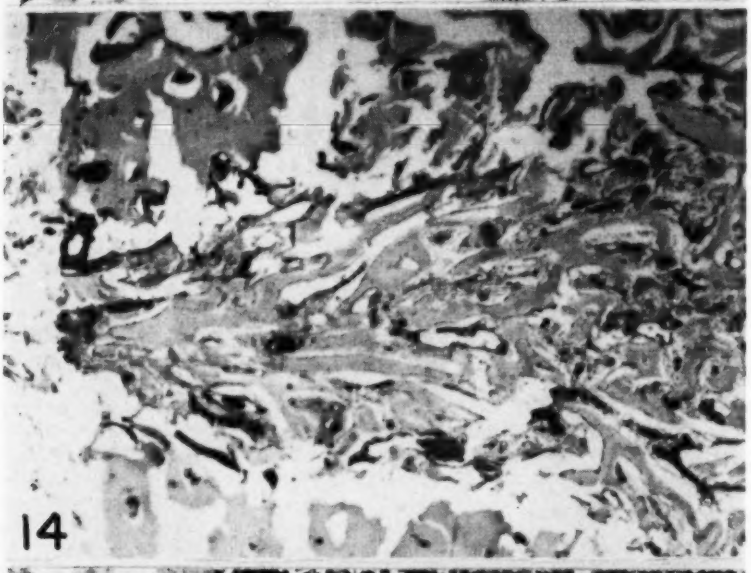


Fig. 14 — Rib drilled fifty-one days before the animal was killed; periosteal surface of the drill hole practically healed over. x 20.

Fig. 15 — Longitudinal section of a rib which had not been subjected to the drill. x 20.

—Esther M. Smith

in different bones, and in different bones at the same level in the same animal. Dameshek [123] contended that for exact cytological study, marrow smears could not be excelled. According to Young and Osgood,[124] the only limitation of aspirated samples is the loss of structural relationships.

Nordenson [83] claimed that marrow from several bones in the same patient was similar in quality and quantity. Williams,[125] studying the cellular pattern of human marrow at autopsy, ascertained that the differential count from different bones in the same case was essentially the same. Jaffé [77] reasoned that the simplicity and lack of technical skill required outweighed the disadvantages of the dilution with blood and failure to give an *in situ* picture. Dameshek, Henstell, and Valentine [126] thought the chief advantage of puncture biopsy, its simplicity, was exceeded by its inaccuracy. Helpap [127] agreed with Custer [122] that bone marrow is not homogeneous and that samples from one part of a bone differ from those from another part. Stasney and Higgins [7] concluded, to the contrary, that there is sufficient uniformity of hematopoiesis in dog marrow so that "the appraisal of the marrow of any one region will reveal what the trend of its cellular changes is elsewhere in the body." A later work by the same authors substantiates this view for human marrow.[28]

Stodtmeister and Buchmann [128] studied the influence of sternal puncture on the circulating blood and found it had no effect on cell composition. Gordon [129] gave as disadvantages of any aspiration method, trauma, the failure to dislodge immature cells, loss of topographic relationships, and the dilution with peripheral blood. Mulligan [130] obtained a favorable correlation by comparing marrow obtained by sternal puncture and trephine methods. Epstein and Tompkins [22] would invalidate differential counts made from smears on the basis of trauma and inadequate distribution of cells. Steiner [131] felt that a marrow aspiration method might not be satisfactory in Hodgkin's disease because of the distribution of the lesions in foci. By comparing marrow smears from the ribs and femurs of dogs, Van Loon and Clark [10] came to the conclusion that such preparations were similar in content. Osgood and Seaman [66] pointed out that any marrow preparation whether a section, an imprint, or aspirated material, will have blood in it because blood is present in the sinusoids and vascular channels of both normal and pathological marrow. According to Schleicher,[132] any kind of sampling from as large and complex an organ as bone marrow is subject to errors of chance.

In spite of all this controversial evidence as to its value, bone marrow examination is a useful tool to be employed when indicated, just as other techniques are. According to Bloom,[133] no hematological study is complete if the bone marrow is neglected.

In reviewing this study and the data presented here, certain points may be noted:

The few stem cells may be readily explained by referring to some investigations of Japa [120] on the mitotic activity of human marrow. He found that out of 100 cells in the myelocytic series only three were myeloblasts and in the erythroblastic system nine were early erythroblasts. Trautmann [134] seldom saw the stem cell in normal bone marrow.

Few young erythroblasts (the proerythroblasts of some authors) are encountered because the multiplication of older cells is sufficient to fill the physiological need.[135]

Most of the mitotic figures occurred in the erythroblastic series. Japa [120] found 15 per thousand nucleated cells in human marrow. The range in the horse was from 0 to 8 per 500 cells counted.

SUMMARY

The normal cytological picture of the bone marrow of the horse has been presented. Samples were obtained from 7 horses. The ribs were chosen as the site for securing the marrow. The samples were obtained by drilling into the rib, inserting a cannula into the drill hole, and aspirating 1 to 2 cc. of marrow. Peripheral blood samples were taken at the same time. Blood and marrow smears were made and stained with Osgood's and Ashworth's [90] modification of Wright's blood stain.

Cell counts were made to establish a "normal" myelogram for the horse. Three hundred cells were counted in the differential leukocyte count on the blood smears and 500 cells were enumerated in the differential count on the marrow smears. The

mitotic figures encountered per 500 cells were also recorded. The megakaryocytes were counted in a 300-sq. mm. area. The myeloid-erythroid ratio was determined. Columns 9 and 10, table 1, summarize these data. Cytological studies were made of the marrow cells and color comparisons were made with colors in the "Munsell Book of Color." [109]

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A Comparison of the Effects of Starvation and Thermal Stress on the Acid-Base Balance of Dairy Cattle

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STARVATION imposes a unique metabolic strain upon dairy cattle; it changes both the origin and the nature of the energy-yielding foodstuffs. The normal base-rich diet of plant origin is supplanted by a base-poor diet, a carnivorous diet the origin of which is the animal's own tissues. Maintenance of the acid-base balance, normally a problem of base elimination in herbivores, becomes, under these conditions, a problem of base conservation and acid elimination.

At high environmental temperatures, dairy cattle attempt to increase evaporative cooling from the respiratory tract by panting; they simultaneously decrease heat production by curtailing feed intake. The panting causes a respiratory alkalosis.[1] The effect of starvation on the acid-base balance of dairy cattle has not been measured.

MATERIALS AND METHODS

Four dairy cattle, 3 Holstein-Friesians and 1 Guernsey, were maintained in the controlled environment of the climatic laboratory[2] at a temperature of 60 to 70 F. and a relative humidity of 60 to 80 per cent. For a six-day control period, these animals were fed a standard dairy ration consisting of alfalfa hay, a grain mixture,* beet pulp, salt, and water; during the five-day starvation period, they were allowed only salt and water, no other food.

Urine was collected with the apparatus described by Hobbs, Hansard, and Barrick.[3] The weight, temperature, and specific gravity of each 24-hour sample were recorded, and a representative sample was retained for chemical analysis.

Plasma for all analyses other than blood pH was separated from jugular blood collected in heparinized flasks. Blood for pH measurement was drawn from the jugular vein into oiled, heparinized syringes. This sample was collected with minimal stasis; exposure to air and agitation were carefully avoided.

Blood pH was measured by the method of Davenport[4] two minutes after the sample was collected. Total ketone bodies in plasma and urine were determined by the method of Greenberg and Lester.[5] Organic acids of urine were measured by the method of Van Slyke and Palmer.[6] The CO_2-combining capacity of the blood plasma was determined manometrically as described in the method outlined by Hawk, Oser, and Summerson.[7] The procedures for the inorganic analyses of blood and urine are to be described in a separate publication.[8]

RESULTS AND DISCUSSION

Figures 1 to 3 show in graphic form the changes in plasma concentration and urine excretion produced by five days of starvation. With the exception of blood pH, these figures are plotted on semilogarithmic paper. These graphs are superior to arithmetic graphs in that on semilogarithmetic paper, equal slopes of the curves represent

TABLE 1 — Plasma Concentration and Urine Excretion of Principal Anions and Cations (Mean Values)

	Plasma concentration mEq./liter		Urine excretion mEq./24 hours	
Constituents	Control period	Last day of starvation	Control period	Last day of starvation
Cation				
Sodium	162.0	157.0	529.0	239.0
Potassium	5.1	4.4	3658.0	485.0
Calcium	7.5	4.8	73.8	13.6
Magnesium	3.2	2.0	374.2	68.3
Total	177.8	168.2	4635.0	805.9
Anion				
Sulfate	14.3	12.1*	759.0	272.0
Phosphate	7.4	7.6	4.2	824.0
Carbonate	25.4	23.5	4500.0	540.0
Chloride			250.0	55.0**
Total	47.1	43.2	5513.2	1691.0

* From third day of starvation; ** value of 678 mEq./liter for Holstein-Friesian 197 not included in calculation of mean.

Portion of a thesis submitted by the senior author to the Graduate School, University of Missouri, in partial fulfillment of the requirements for the degree of doctor of philosophy.

Journal series paper 1398 approved by the director of the Missouri Agricultural Experiment Station.

From the Department of Veterinary Physiology and Pharmacology (Dale); Department of Agricultural Chemistry (Goberdhan); Department of Dairy Husbandry (Brody).

The authors gratefully acknowledge the coöperation and advice of Drs. Charles W. Gehrke and E. E. Pickett of the Experiment Station Analytical and Spectrographic Laboratories and their help in conducting some of the analytical chemical work.

* The grain mixture was composed of 66 parts of corn, 66 parts of oats, 35 parts of bran, 30 parts of soybean oil meal, 3 parts of sodium chloride, and 2 parts of steamed bone meal; these ingredients were ground and thoroughly mixed.

equal relative or (when multiplied by 100) equal percentage changes of the functions under comparison. Semilogarithmic graphs also present a much wider range of concentrations than do the usual arithmetic graphs. Table 1 shows the mean values of plasma concentration and urine excretion during the control period and on the last day of starvation.

The cations, sodium, potassium, calcium, and magnesium (fig. 1), show a similar pattern; the plasma concentrations are changed slightly and the urinary excretions are changed profoundly. In milliequivalents per liter (table 1), total cations of plasma fell from an average of 177.8 during the control period to an average of 168.2 on the last day of starvation, a drop of 5.4 per cent. During the same period, urinary excretion of total cations dropped from an average of 4,635 mEq. per twenty-four hours to an average of 806 mEq. per

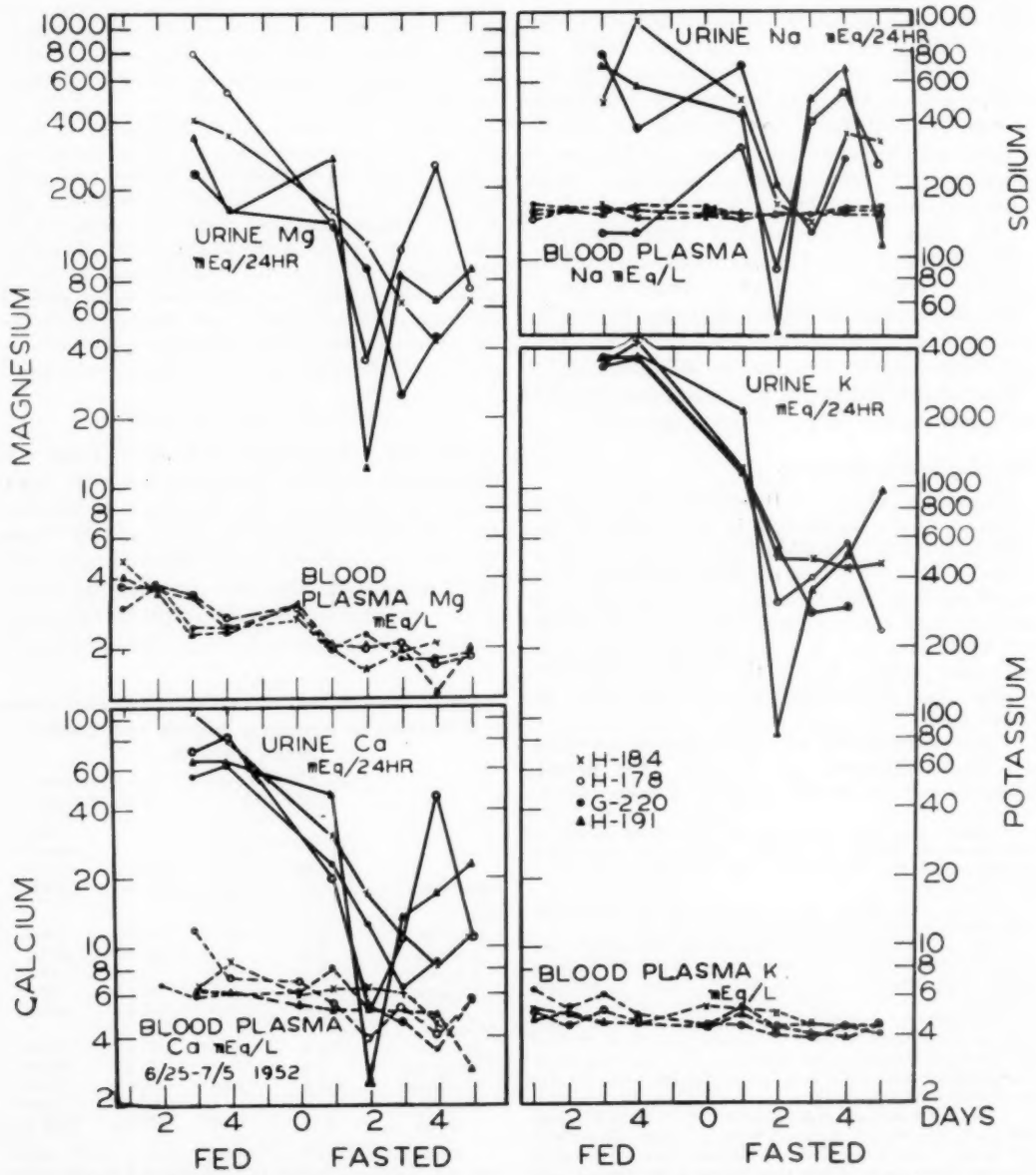

Fig. 1—Cations in plasma and urine; changes produced by five days of starvation.

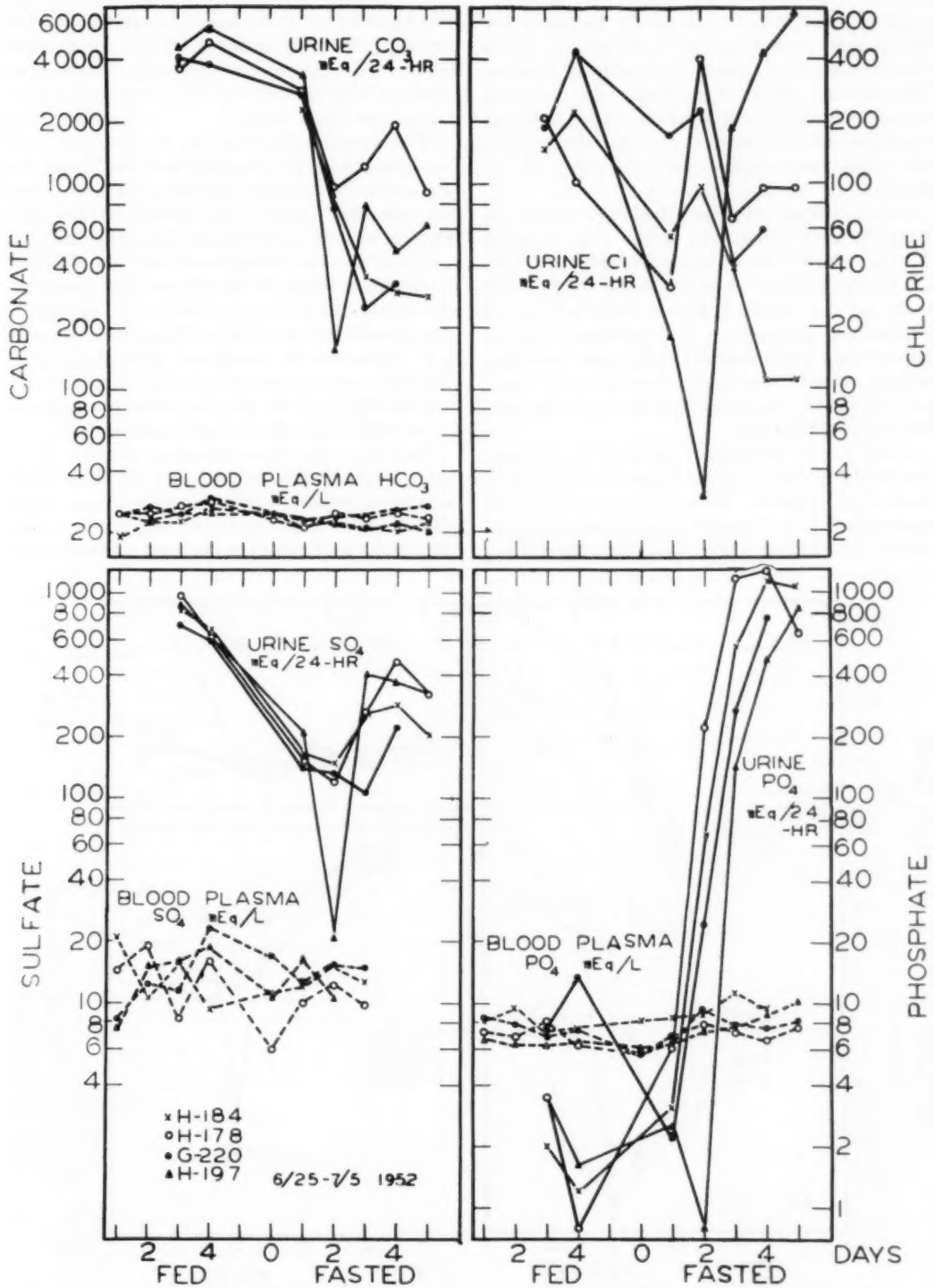

Fig. 2—Anions in plasma and urine; changes produced by five days of starvation.

twenty-four hours, a decline of 82.6 per cent. These changes, especially the decrease in urinary excretion, are a reflection of the base content of plant and animal tissues. The maintenance of a relatively constant blood concentration despite overwhelming changes in urinary excretion dramatizes the remarkable homeostatic function of the kidney.

Total anions appear similar to total cations in both blood and urine (fig. 2, table 1). Expressed as milliequivalents per liter of plasma, there is a decrease of 8.3 per cent in the total of the concentrations of carbonate, phosphate, and sulfate between the control period and the last day of starvation. Simultaneously, there is a 69.3 per cent decrease in total anions excreted per twenty-four hours.

Most of the decrease in anionic excretion, however, is due to a decrease in the excretion of carbonate. This ion is available for excretion in unlimited quantities; its concentration in the urine measures the difference between the amount of fixed acids and the amount of fixed bases available for excretion. Two factors contribute to the observed decrease in urinary carbonate, namely a decrease in fixed base and an increase in fixed acid.

The remarkable rise in the excretion of inorganic phosphate results from tissue destruction for energy purposes. The organic phosphorus compounds, phosphatides, nucleic acid and nucleotides, phosphocreatine, and the hexose phosphates, are catabolized with the release of inorganic phosphate in the blood and the excretion of inorganic phosphate in the urine. The comparatively low excretion of inorganic phosphate during the control period results from the unavailability of the phytic acid phosphorus in cereals and other plant material.

Sulfate excretion which decreased to a low value on the second day of starvation and then returned to an intermediate value shows the transition from exogenous to endogenous metabolic mixtures. Apparently

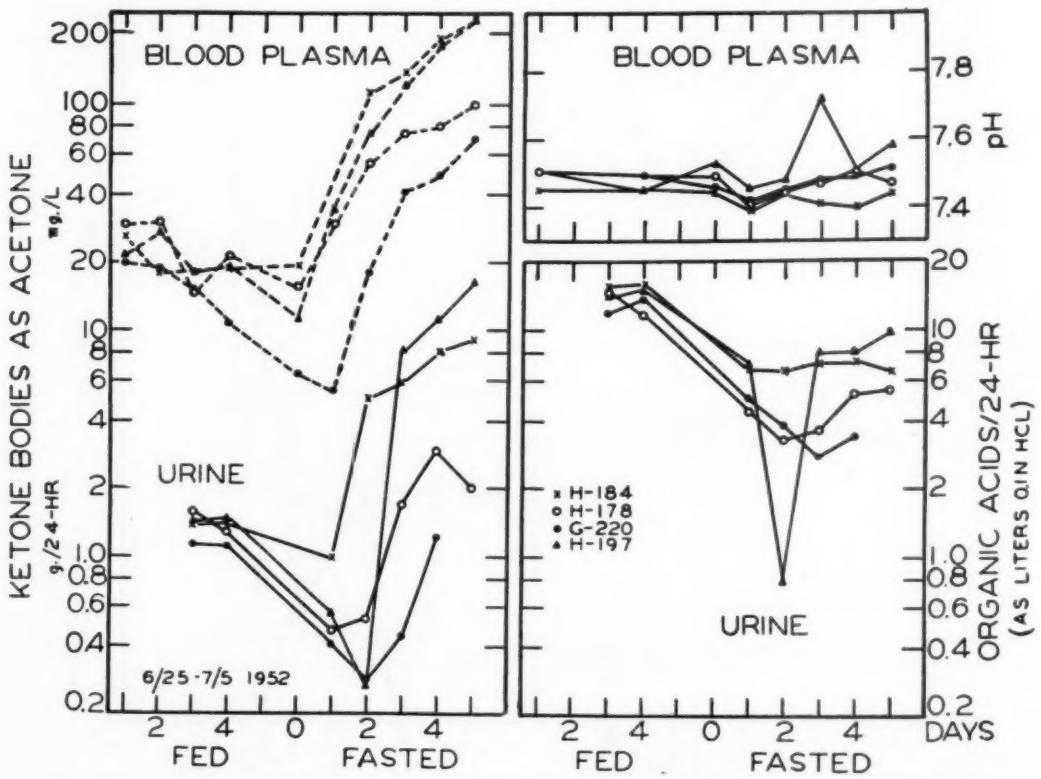

Fig. 3—Total ketone bodies in plasma and urine, organic acids in urine, and blood pH; changes produced by five days of starvation.

the sulfur-containing amino acids are more abundant in the feed consumed by the herbivorous animal than they are in the tissues of that animal.

In contrast to the effects of high temperature,[1] blood pH* (fig. 3) remained relatively constant during the five days of starvation. This lack of significant change and the uniformity of the plasma bicarbonate indicate that starvation plays little or no part in the disturbance of the acid-base balance produced by thermal stress.

The 24-hour excretion of total ketone bodies in the urine appears directly proportional to the concentration of total ketone bodies in the blood plasma (fig. 3). This relationship, different from that of any other substance studied, resembles the blood concentration and urinary excretion of glucose in pancreatic diebetes. The ketone relationship differs from blood and urine glucose in that there is a considerable excretion of ketone bodies when the concentration of ketone bodies in the blood is normal. Lactation** had little or no effect on the development of a starvation ketosis; Holstein-Friesian 178, the highest-producing cow, was second lowest in terms of the blood concentration and urine excretion of total ketone bodies.

The excretion of organic acids in the urine (fig. 3) was decreased by starvation. Since ketone bodies are included in the organic acids, there was some organic fraction that underwent more of a decrease than is apparent in figure 3. It is suggested that the quantity of organic acids excreted in the urine may be proportional to the absorption of organic acids from the rumen.

* Due to improper standardization, all pH readings are approximately 0.15 pH units higher than the actual value.

** Holstein-Friesian 178 produced an average of 27.5 lb. of milk per day during the control period and produced 5.1 lb. on the last day of starvation. Holstein-Friesian 184 produced an average of 9.0 lb. of milk per day during the control period and 0.5 lb. on the last day of starvation. Holstein-Friesian 197 and Guernsey 220 were not lactating.

SUMMARY AND CONCLUSIONS

Five days of starvation produced only slight changes in the concentration of the principal inorganic anions and cations in the plasma; however, five days of starvation resulted in massive changes in the urinary excretion of these substances. Changes of the most significance were the decreased excretion of cations and carbonate and the increased excretion of phosphate.

Distortions of the acid-base balance of the blood characteristic of exposure to thermal stress were not produced by starvation. Blood pH and bicarbonate content showed no significant change; the concentration of total ketone bodies in the blood plasma and the excretion of total ketone bodies in the urine were increased; and the excretion of organic acids in the urine was decreased. These alterations, all produced by starvation, do not result upon exposure to thermal stress; the two conditions, heat and starvation, apparently have separate and distinct effects on the acid-base balance of the blood.

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The Rumenitis-Liver Abscess Complex in Beef Cattle

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INFLAMMATION of the stomachs and abscesses of the liver constitute a complex of lesions in which gastric injuries are primary and hepatic abscesses are secondary.

Hepatic abscesses are responsible for economic losses to packers by necessitating the condemnation of affected livers at slaughter and to producers of cattle by causing occasional deaths and by reducing efficiency in utilization of food materials. Rumenitis probably lowers efficiency of ruminal digestion during fattening and causes condemnation of affected rumens at slaughter.

This study was made with the following objectives: (1) to determine the relation of lesions of the rumen, reticulum, and abomasum to abscesses of the liver; (2) to determine gross and microscopic pathology of lesions of the rumen of fat cattle; and (3) to determine the ability of the products of fermentation of grain within the rumen to produce, under experimental conditions, lesions of the rumen similar to lesions which occur naturally.

REVIEW OF LITERATURE

Newsom [6] and Madin [5] in the United States showed that approximately 85 per cent of abscessed livers from fat beef cattle contained a pure infection of *Spherophorus necrophorus* and that an additional 10 per cent contained *S. necrophorus* plus other species of bacteria. Yamamoto [10] in Japan found *S. necrophorus* in bovine liver abscesses.

Robinson, Jasper, and Guilbert [8] injected into rabbits, in 2-ml. quantities, strained contents from rumens of 8 normal steers. By this means, *S. necrophorus* was isolated from 7 steers.

From the School of Veterinary Medicine (Jensen, Miller, and Graham) and the Department of Zoölogy (Cooper), Colorado A. & M. College, Fort Collins. From the Army of the United States (Lt. Col. Deane, V.C.). A portion of these data was supported by a Ralston Purina Research Fellowship and was contained in a thesis submitted by the senior author to the Graduate School, University of Minnesota, in partial fulfillment of requirements for the degree of doctor of philosophy.

The meat-packing companies of Denver, Colo., especially Wilson and Company, and Swift and Company, cooperated in this study by extending use of facilities and personnel. Dr. J. W. Hovorka, inspector in charge, Bureau of Animal Industry, and his staff were helpful in the examination of healthy and diseased organs and in permitting the removal of diseased tissue to the laboratories at Colorado A. & M. College, Fort Collins.

Jensen, Flint, and Griner [4] reproduced liver abscesses experimentally in cattle and sheep by intraportal inoculation of viable *S. necrophorus*.

Hutyra, Marek, and Manninger [3] indicated that necrotic rumenitis occurred commonly in cattle. The foci of necrosis varied in size and shape. They were firm, brown, and extended through the mucosa. The cause was injury to the mucosa by sand, tuberculosis, cattle plague, viruses of foot-and-mouth disease and malignant catarrhal fever, and penetration by foreign bodies. *Spherophorus necrophorus*, which was always present, was able to penetrate injured tissue, grow, and cause necrosis. The lesions might have resulted in motor weakness of the rumen.

The Denver laboratory of the United States Bureau of Animal Industry [7] indicated that abscesses of the liver were sometimes associated with ulcers of the rumen.

Smith [9] studied the relation of ruminal ulcers to abscesses of the liver in cattle. Livers and stomachs of 1,807 fat cattle were examined. Three hundred and twenty-two contained abscessed livers; of these, 201 also contained ruminal lesions. Of the 1,485 cattle with normal livers, 273 presented ruminal lesions. It was concluded that ruminal lesions were associated with liver abscesses. Smith considered ruminal lesions to be related but classified them into eight types: (1) adherent contents; (2) denuded, depigmented, and eroded areas; (3) ulcers having pseudodiphtheritic membranes; (4) clean ulcers; (5) scars; (6) papillomatous proliferations; (7) clumped villi; and (8) submucous nodules.

Hungate, Dougherty, Bryant, and Cello [2] studied fermentation in the rumen. Using sheep as experimental animals, rye, wheat, and glucose were placed with water in the rumen. Fermentation proceeded rapidly. The pH of the ingesta fell to 4.1 to 4.7. Rumination ceased and respiration was accelerated. Some animals died after being fed the material. *Streptococcus bovis* increased rapidly and presumably caused the fermentation. Acetic, propionic, butyric, and lactic acids were identified in the ruminal contents. The titrable acidity was as much as 0.13 mEq. per milliliter of ingesta.

Cohrs [1] classified defects in the mucosa of the abomasum as: (1) erosions which extended into the mucosa only, and (2) ulcers which extended through the mucosa into deep layers of the gastric wall.

MATERIALS AND METHODS

To obtain information on the pathogenesis of hepatic abscesses in cattle, the following phases of

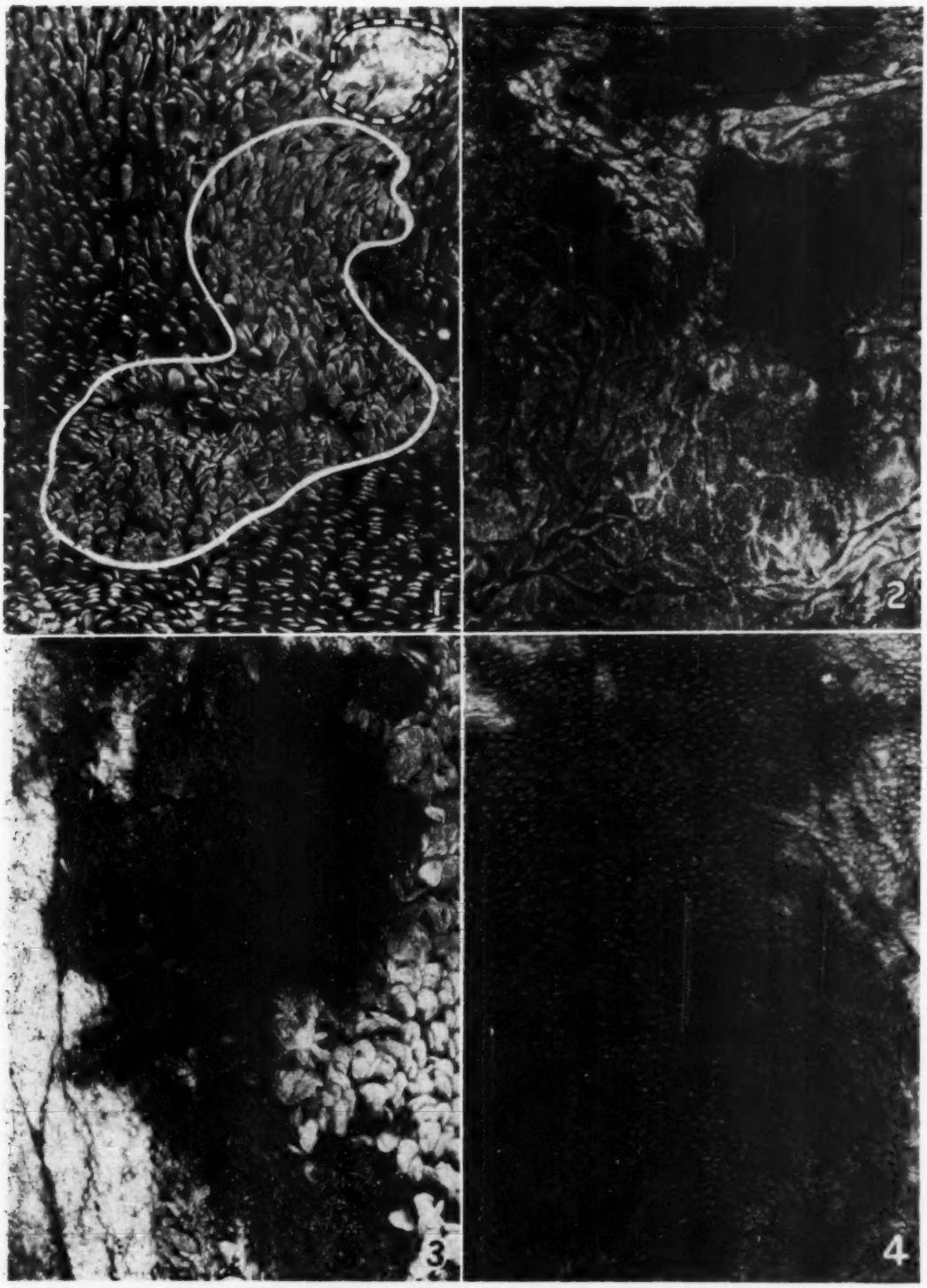

Fig. 1—Acute rumenitis of the anterior dorsal sac. Solid line encircles necrotic villi. Broken line encircles area of devillation.

Fig. 2—Acute rumenitis. Mucosa is necrotic, hemorrhagic, and devillated. Anterior ventral sac of rumen.

Fig. 3—Closer view of figure 2.

Fig. 4—Acute rumenitis of anterior ventral sac. Mucosa is edematous, necrotic, and devillated.

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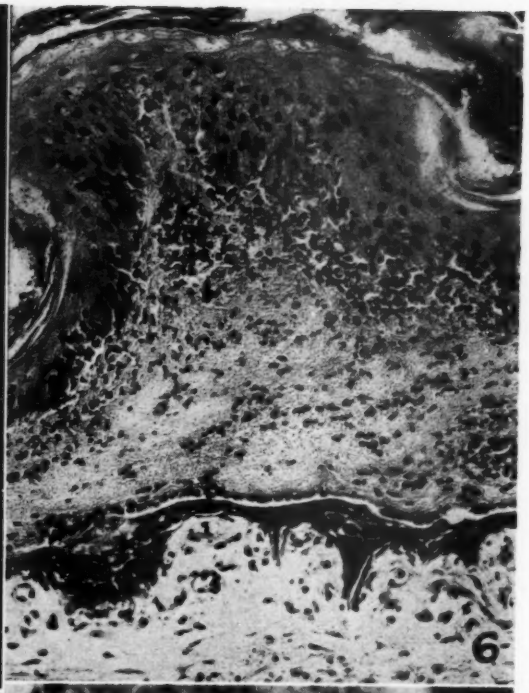
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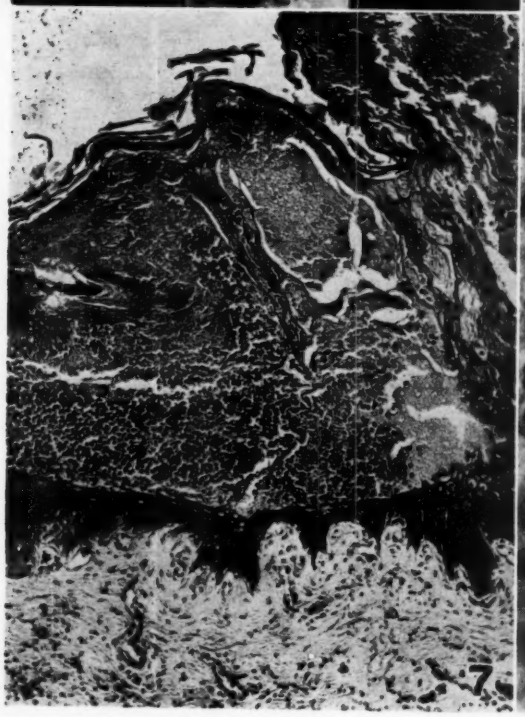
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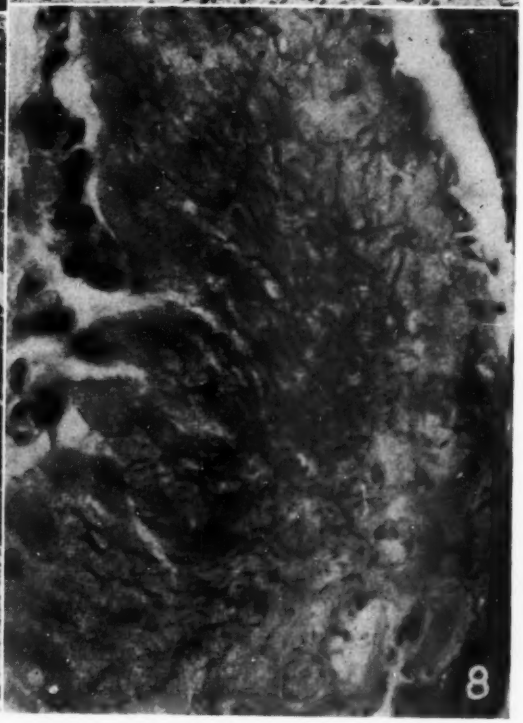
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the disease were studied: (1) the relation of the lesions of the stomachs to abscesses of the liver and (2) experimental rumenitis.

RELATION OF LESIONS OF THE STOMACHS TO ABSCESSES OF THE LIVER

Fattened Adult Cattle. — Source. — Livers and stomachs from 1,535 cattle were examined. All types of cattle were included in the sample; however, the majority was healthy Hereford steers and heifers of good quality and of ages from 9 to 24 months. The animals were slaughtered by Wilson and Company, Denver, from January through May, 1952. Eighty-four lots, constituting all animals slaughtered each Thursday between 7:00 a.m. and 12:00 p.m. were examined.

TABLE 1—Classification and Incidence of Spontaneous Gastric Lesions in 1,535 Adult, Fat Cattle

Type of lesion	Incidence	
	No. affected	Total sample (%)
Normal stomachs	694	45.2
Injured stomachs	841	54.8
Inflammation of forestomachs	662	43.1
Rumenitis	577	37.6
Acute	37	.....
Chronic	540	.....
Traumatic reticulitis	85	5.5
Hyperkeratosis of rumen and reticulum	135	8.8
Adherent contents	85	.....
Clumped villi	50	.....
Ulcers of abomasum	44	2.9

Examinations at the Packinghouse.—Livers were examined for abscesses and other abnormalities. Rumens, reticulums, and abomasums were opened longitudinally, cleaned, and examined for abnormalities. All lesions were located anatomically and classified as to type.

Processing at the Laboratory.—All gastric lesions were excised, transported to the laboratory, and fixed in 4 per cent formaldehyde. Sections were stained with hematoxylin and eosin, Nocht's stain, and McCallum-Goodpasture Gram's stain.

Calves Not Fattened.—Livers and stomachs of 630 calves were examined. The animals were mostly of beef breeds, recently weaned, and weighed 200 to 500 lb. All calves slaughtered by Swift and Company, Denver, on Tuesdays and Thursdays of October, 1952, were examined. Examination of livers and stomachs was performed in the same manner as was employed on adult cattle.

EXPERIMENTAL RUMENITIS

Experiment 1.—Three young adult cattle, which had been maintained on a ration of alfalfa, were fed barley forcibly as indicated in table 6. At autopsy, contents of the forestomachs were collected for pH determinations. Walls of the forestomachs were examined for lesions.

Experiment 2.—Six young adult cattle, which had been maintained on a ration of alfalfa, were arranged in three pairs. Barley, acetic acid, and sodium acetate were fed separately to the pairs of animals as indicated in table 7. Samples of rumen contents were collected daily beginning several days prior to, and continuing throughout, the period of experimental feeding. At autopsy, walls of the forestomachs were examined for lesions.

RESULTS

Spontaneous gastritis and liver abscesses occurred commonly in adult fattened cattle and uncommonly in unfattened calves. Experimental rumenitis, which resembled the natural disease, was produced within the rumen by fermentation of barley.

RELATION OF LESIONS OF THE STOMACHS TO ABSCESSES OF THE LIVER IN FATTENED CATTLE

Based on gross and histopathological changes, table 1 presents types and incidence of stomach lesions.

Rumenitis.—Nontraumatic inflammation constituted 68.6 per cent of all stomach lesions. Both acute and chronic stages were observed. The majority of lesions was of long duration.

Gross Pathology.—Acute rumenitis was characterized by hyperemia, hemorrhages, edema, gas formation, and necrosis. Hyperemia and hemorrhages were uneven in distribution and conspicuous in mucosae which contained little pigment. Edema was manifested by the presence of transudate which was discernible in the mucosa

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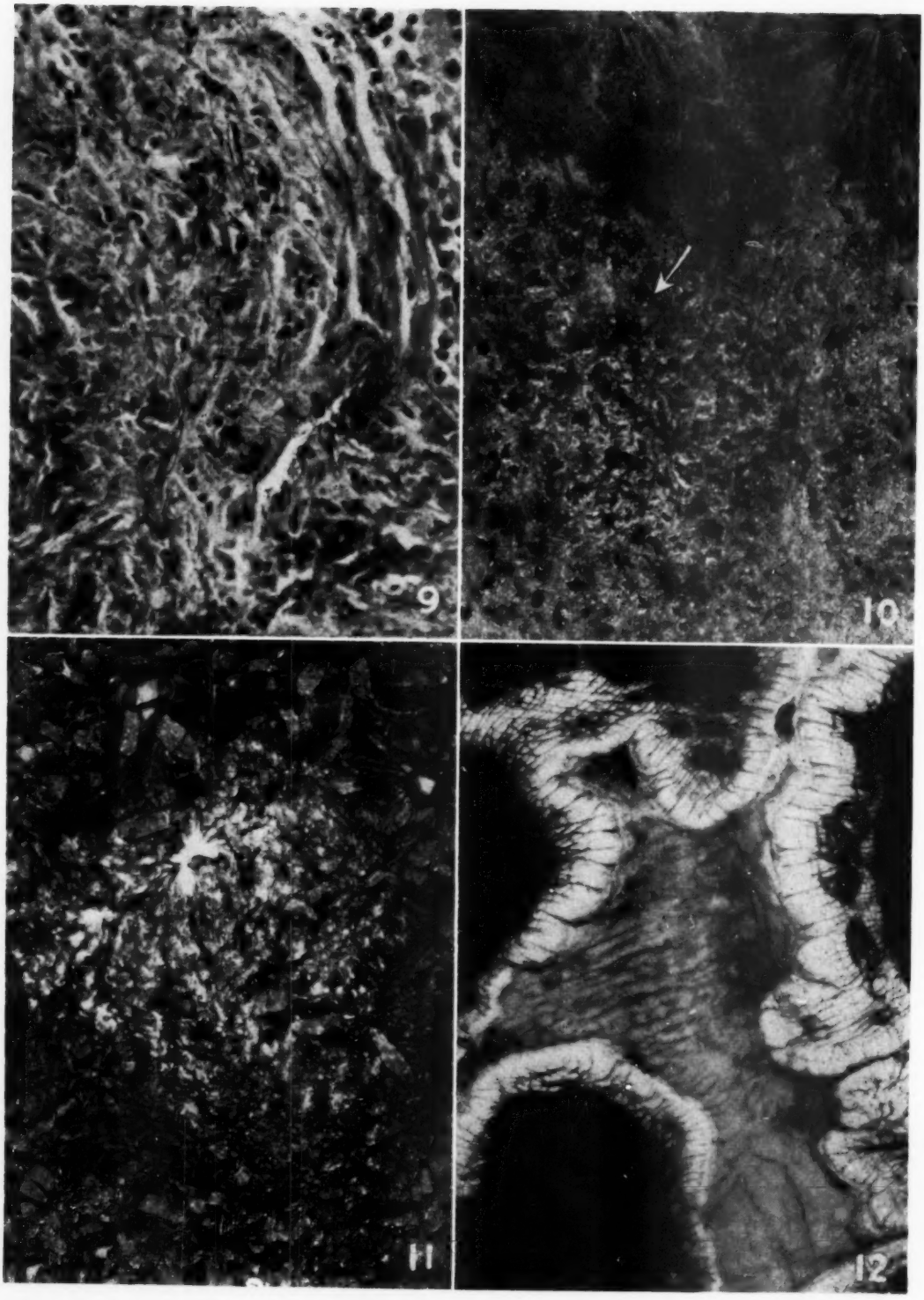
Fig. 5—Acute rumenitis. Cross section of wall of anterior ventral sac. Arrow points to necrosis and gas.

Fig. 6—Acute rumenitis of anterior ventral sac. Spinose layer of epithelium is vesicated; superficial layers of cells are necrotic. Nocht's stain. x 200.

Fig. 7—Acute rumenitis of anterior ventral sac. Epithelium is vesicated and necrotic. Nocht's stain. x 100.

Fig. 8—Acute rumenitis of anterior ventral sac. Epithelium is necrotic and a colony of Spherophorus necrophorus is growing into the dead tissue. Nocht's stain. x 900.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from the intact surface, and in the submucosa from gross sections. Affected tissues were thickened. Edema was accompanied often by the presence of multiple gas bodies, some as large as 2 mm. in diameter.

Necrotic tissue was friable and gray unless darkened by extravasated blood. Villi were swollen and easily detached. The shape of necrotic foci was irregular, and the size varied from minute to 5 cm. in diameter. Dead tissue extended into the submucosa. Common locations were the anterior ventral and anterior dorsal sacs. Figures 1 to 5 show acute rumenitis.

Chronic rumenitis was characterized by devillation, depigmentation, scars, thickening of mucosa and submucosa from fibrosis, pits in the epithelium, nodules, and ulcers. Devillated areas, found commonly in the anterior ventral and anterior dorsal sacs, varied in size from a few millimeters to 20 cm. in diameter. Scars and ulcers were often within the devillated area. In the anterior dorsal sac, devillation frequently was without complications. Lesions of long duration contained regenerating villi, especially at the periphery.

Some pigmented rumens were depigmented in foci which were irregular in shape and variable in size. Some foci were as large as 15 cm. in diameter. In case of complete loss of pigment, the affected tissue was conspicuous. This change was accompanied usually by devillation, scars, and ulcers, and was located commonly in the anterior ventral sac.

Scars were abundant. Their shape, size, and number varied. As many as 75 were found in a single rumen. The majority of scars was located in the anterior ventral and anterior dorsal sacs.

Depressions, or pits, in the epithelium were single or multiple and measured 1 to 2 mm. in diameter and approximately 1 mm. in depth. Some were dark in color because of extravasated blood. The periphery of some pits was hyperkeratinized, causing severe roughness of the mucosal surface. Devillation and depigmentation often were concurrent.

Nodules were of two types—epithelial and fibrous. Epithelial nodules were single or multiple, spherical masses imbedded in mucosa. They were gray to white, firm, and measured 5 mm. or less in diameter. They projected slightly onto the mucosal surface and were enucleated readily by digital pressure. Fibrous nodules formed small pliable, pedunculated masses which projected onto the surface and were ulcerated in some cases. Most nodules were located in the anterior ventral sac.

Denuded areas were single or multiple, irregular in outline, and measured as much as 6 cm. in diameter. The majority of such defects extended into the submucosa. Many scars were ulcerated. Exudate, necrotic tissue, and ingesta accumulated in the crater. When washed, the denuded tissue was smooth and pink. Epithelium was regenerated from the periphery and from islands of epithelium within the ulcer. Anatomically, ulcers were found in the anterior dorsal and anterior ventral sacs and on the ruminal pillars. Figures 10 to 18 show chronic rumenitis.

Histopathology.—In acute inflammation, the epithelium was necrotic. The spinose layer contained vesicles which were commonly filled with leukocytes. The superficial covering of the vesicle was necrotic while the deep part was viable. Bacteria, including *S. necrophorus*, were observed in the vesicles and necrotic epithelium. The submucosa presented all changes which characterize acute inflammation. Exudate consisted of fibrin, serum, and neutrophils. Necrosis was extensive and many vessels were thrombosed. Bacteria, including *S. necrophorus*, were in affected submucosa. Gas bodies were present. The histopathology of acute rumenitis is shown in figures

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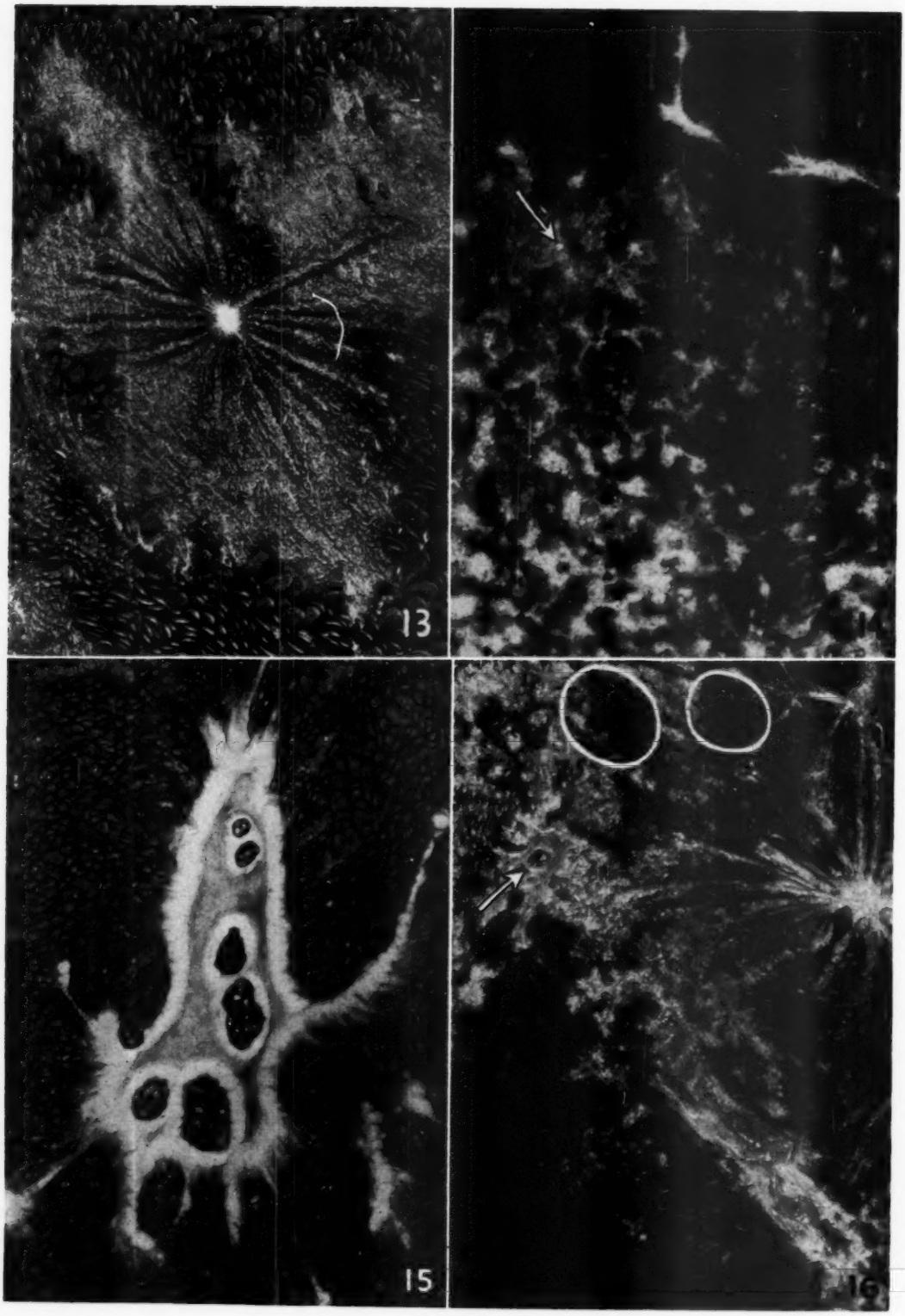
Fig. 9—Acute inflammation of the submucosa of anterior ventral sac of the rumen. Spherophorus necrophorus has penetrated into the submucosa. Tissue is necrotic. Nocht's stain. x 400.

Fig. 10—Chronic rumenitis of anterior ventral sac. Mucosa contains scars and pits which are remains of old vesicles. Villi have been destroyed. Arrow points to old vesicle.

Fig. 11—Chronic rumenitis of anterior dorsal sac. Mucosa is devillated, depigmented, and scarred.

Fig. 12—Ulcer from chronic rumenitis of anterior pillar. Mucosa is depigmented and ulcerated.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6 to 9. In lesions of chronic rumenitis, the epithelium was thickened especially in stratum corneum and stratum spinosum. Some rete pegs were lengthened to extend into the submucosa, and contained epithelial nodules which consisted of concentric layers of keratinized squamous cells. The spinose layer contained empty cavities of collapsed vesicles. At the periphery of the lesions, some villi were regenerating. The submucosa was thickened with granulation tissue. Collagenous fibers were hyalinized. Eosinophils, lymphocytes, and mononuclear macrophages were abundant. Multinucleated giant cells accumulated in and around lymphatics. The histopathology of chronic rumenitis is shown in figures 19 to 21.

TABLE 2—Anatomical Location and Incidence of Lesions in the Rumens of 1,535 Adult Cattle

Location of lesions	Incidence	
	No. affected	Total sample (%)
Rumenitis	577	.....
Anterior dorsal sac	51	8.8
Anterior dorsal sac + anterior ventral sac	105	18.2
Anterior ventral sac	410	71.1
Anterior ventral sac + pillars	11	1.9
Hyperkeratosis	135	.....
Dorsal curvature	75	55.6
Dorsal curvature + anterior ventral sac	6	4.4
Anterior dorsal sac	25	18.5
Anterior dorsal sac + others	5	3.7
Anterior ventral sac	11	8.1
Anterior ventral sac + anterior dorsal sac	4	3.0
Posterior dorsal sac	9	6.7

Anatomical Location of Rumenitis.—Table 2 shows anatomical location and incidence of ruminal lesions.

Traumatic Reticulitis.—Table 1 shows that traumatic reticulitis constituted 12.8 per cent of the total cases of inflammatory reaction in the forestomachs.

Ingesta in the stomachs contained many nondigestible materials which were capable of traumatism. These included glass, wood, plastics, wire, nails, hair pins, metallic buckles, pieces of coal, and stones measuring as much as 4 cm. in diameter. Metallic bodies tended to accumulate in the lumen of the reticulum.

Hyperkeratosis.—Hyperkeratosis, an accumulation of excessive layers of keratinized cells in the stratum corneum, was subdivided into two forms—adherent contents and clumped villi. Incidence of hyperkeratosis is shown in table 1.

Gross Pathology.—"Adherent contents" was a type of hyperkeratosis characterized by the presence on the surface of the mucosa of dark brown scales to which ingesta adhered. The scales were firm and friable and measured 1 to 2 cm. in diameter. Anatomically, they occurred along the dorsal curvature of the rumen. On removal of the scale, the remaining mucosa was intact but marked by a gray spot where the scale had been attached. Many isolated villi were hyperkeratotic throughout all or a portion of the villus. "Clumped villi" was a type of hyperkeratosis characterized by adherence of five to 20 villi to form a clump.

TABLE 3—Classification and Incidence of Injuries of the Mucosa of the Abomasums in 1,535 Adult Cattle

Type of lesion	Incidence	
	No. affected	Total sample (%)
Erosions	677	44.1
Fundus	281	.....
Pylorus	308	.....
Fundus + pylorus	88	.....
Ulcers	28	1.8
Fundus	12	.....
Active	8	.....
Scars	4	.....
Pylorus	16	.....
Active	7	.....
Scars	9	.....
Erosions + Ulcers	16	1.0

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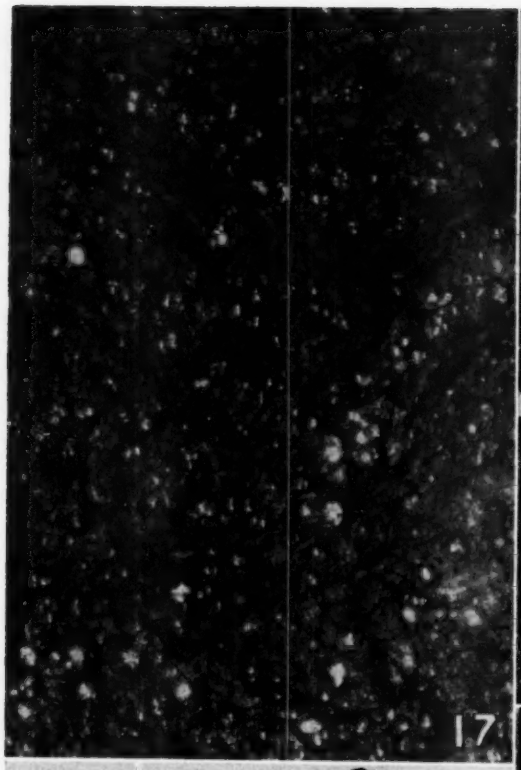
Fig. 13—Chronic rumenitis of anterior dorsal sac. Mucosa is devillated and scarred at center.

Fig. 14—Chronic rumenitis of anterior ventral sac. Mucosa is devillated, depigmented, and scarred, and contains multiple epithelial nodules. Arrow points to epithelial nodule.

Fig. 15—Chronic rumenitis of anterior ventral sac. Mucosa is ulcerated. Epithelium is regenerating at periphery and from islands of persisting epithelium.

Fig. 16—Acute rumenitis superimposed on chronic rumenitis in anterior ventral sac. Mucosa is devillated, depigmented, ulcerated, scarred, and in foci necrotic. Solid lines encircle areas of necrosis. Arrow points to ulcer.



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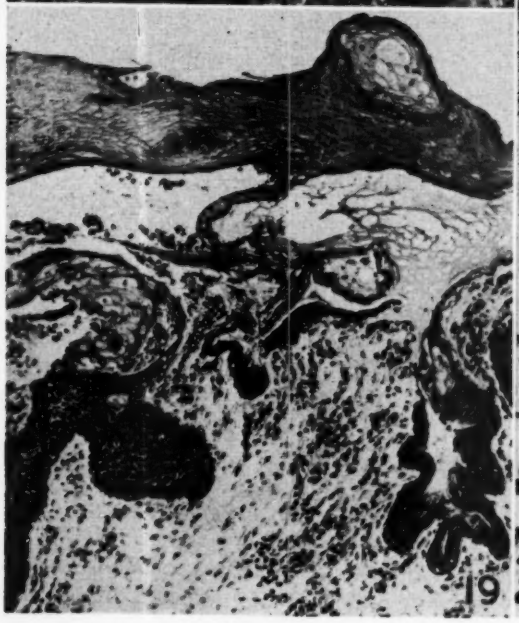

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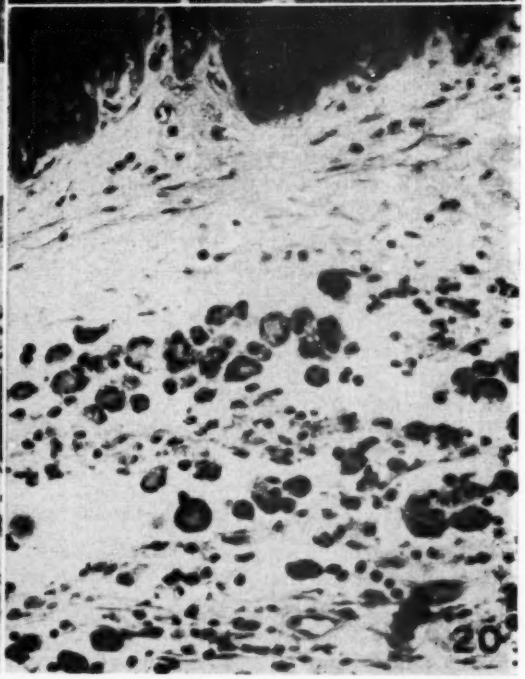

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Some ingesta adhered to the affected villi. By manipulation or forcible washing, individual villi were separated from the clump. When separated, they were thickened but intact. Anatomically, the clumps occurred in the anterior dorsal sac and occasionally in other villated areas. Figure 22 shows hyperkeratosis.

Histopathology. — Microscopically, both forms of hyperkeratosis, adherent contents and clumped villi, presented an accumulation of excessive layers of keratinized cells in the stratum corneum. Superficial layers contained particles of ingesta and colonies of bacteria, *S. necrophorus* not being identified. Figure 23 shows microscopic features of hyperkeratosis.

Lesions of the Abomasum.—Tables 1 and 3 show the classification of erosions and ulcers and their incidence.

Association of Gastric Lesions with Abscesses.—Table 4 shows the association of general gastric lesions with liver abscesses.

The *chi*-square test of independence was highly significant at the 1 per cent level for the association of abscessed livers with injured stomachs and normal livers with normal stomachs.

Relation of Lesions of the Stomachs to Abscesses of the Livers in Unfattened Calves

Young cattle which had not resided in fattening pens contained a low incidence of gastritis and lower incidence of liver abscesses. Examination showed that 7.46 per cent of unfattened calves had injuries in the forestomachs and 0.48 per cent contained abscessed livers. The abomasums showed an incidence of 35.1 per cent of erosions and ulcers. Table 5 presents the incidence of types of lesions in anatomical parts of all stomachs.

Experimental Rumenitis

In the rumens of cattle, unaccustomed to grain as a part of their ration, barley fermented and produced low pH values and severe rumenitis.

Experimental Feeding of Barley to Cattle in Experiment 1.—Details of this investigation are presented in table 6.

TABLE 4—General Stomach Lesions and Liver Abscesses in 1,535 Adult Fat Cattle

	Incidence	
	No. affected	Total sample (%)
Stomachs injured	841	.....
Livers abscessed	341	40.5
Livers normal	500	59.5
Stomachs normal	694	.....
Livers abscessed	156	22.5
Livers normal	538	77.5

Experimental Feeding of Barley, Acetic Acid, and Sodium Acetate to Cattle in Experiment 2.—Details of this investigation are presented in table 7. Animals 2-741 and 2-745, which received barley, showed loss of ruminal motility after the second feeding and accelerated respiration toward the end of the experiment. Body temperatures remained normal.

Gross Pathology.—Rumens of animals fed barley showed generalized inflammation and necrosis. The villi were dark, swollen, necrotic, and friable, especially in the anterior ventral sac. Devillation was developing. Rumens of 2 animals presented edema and gas in the submucosa. Except for generalized distribution, the changes were similar to cases of spontaneous acute rumenitis of fat cattle.

Histopathology.—The epithelium of animals fed barley was necrotic and vesicated. Parts of or entire villi were necrosed. The vesicles contained serum, erythrocytes, leukocytes, and colonies of bacteria including *S. necrophorus*. In some areas, bacteria had penetrated through necrotic epithelium into the submucosa. These changes were similar to histopathology of spontaneous acute rumenitis of fat cattle. Figures 24 to 30 show experimental rumenitis.

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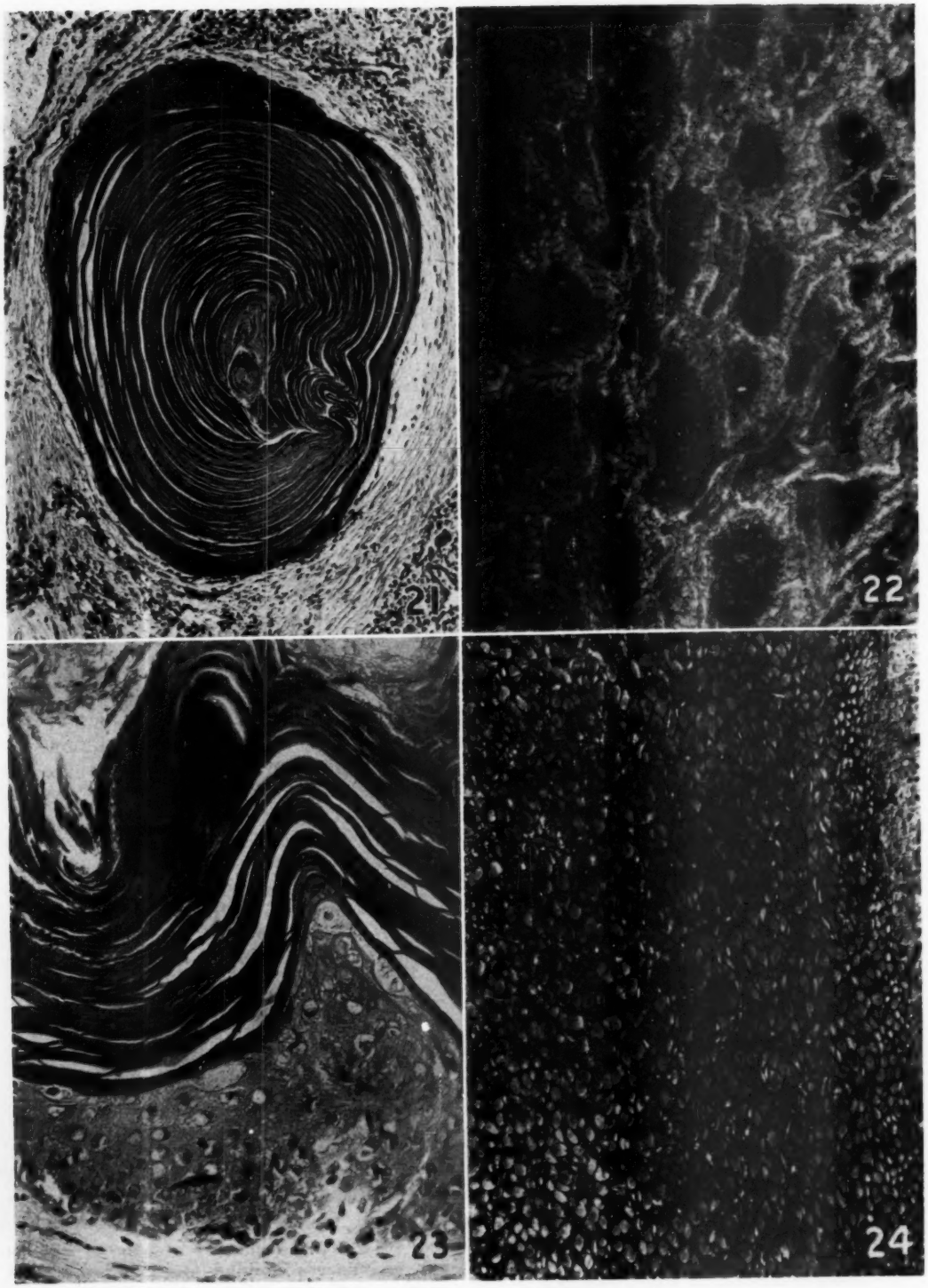
Fig. 17—Chronic rumenitis of anterior ventral sac. Mucosa and submucosa contain multiple epithelial nodules.

Fig. 18—Chronic rumenitis of anterior ventral sac. Mucosa contains multiple pedunculated fibrous connective tissue nodules.

Fig. 19—Chronic rumenitis of anterior ventral sac. Epithelium contains collapsed vesicles which appear grossly as pits. Leukocytes have infiltrated the submucosa. Nocht's stain. x 100.

Fig. 20—Chronic rumenitis of anterior ventral sac. Submucosa contains multinucleated giant cells in and around lymphatics. Nocht's stain. x 200.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DISCUSSION

Because lesions of necrobacillosis were limited to the liver, and because *S. necrophorus* had been found in the alimentary tracts of healthy cattle, it was logical to search for lesions in the alimentary tract through which *S. necrophorus* could penetrate into the portal blood. Of the 1,535 adult cattle studied, 54.8 per cent contained lesions in the stomachs. If these lesions constituted potential points of entrance into the portal system, it is not surprising that 32.3 per cent of the same series contained liver abscesses. Smith's[9] series of 1,807 cattle contained 26.2 per cent of rumenitis and 17.8 per cent of liver abscesses. The *chi*-square test of independence applied to Smith's data indicated high statistical significance for the association of rumenitis and liver abscesses. Many individual animals contained multiple liver abscesses without demonstrable lesions in the stomachs. Since the abscesses may endure for 150 days, it is possible that rumenal lesions gave origin to the abscesses and that they had healed by the time of slaughter. It is also possible that the points of entrance into the portal system were posterior to the stomachs and consequently were not observed.

TABLE 5—Liver Abscesses and Gastric Lesions in 630 Calves

Type of lesion	Incidence	
	No. affected	Total sample (%)
Livers abscessed	3	0.48
Rumens	....	....
Rumenitis	22	3.5
Hyperkeratosis	24	3.8
Reticulums injured traumatically	1	0.16
Abomasums injured	221	35.1

TABLE 6—The Feeding of Barley to 3 Cattle in Experiment 1

Animal (No.)	Experiment (days)	Barley fed (lb.)	pH-ruminal content terminal	Condition of rumen
2-844	1	20		
	2	20		
	4	20		Severe
	7	20	4.2	rumenitis
2-1736	1	10		
	2	10		
	4	10		
	6	10		Severe
	8	10	4.1	rumenitis
2-1737	1	7		Severe
	2	7	4.2	rumenitis

TABLE 7—Force Feeding of Barley, Acetic Acid, and Sodium Acetate to Cattle Which Were Unaccustomed to These Materials

Animal (No.)	Feed	pH of ingesta	Terminal condition of rumen
2-741	Barley 6 lb./day for 2 days	3.99	Rumenitis
2-745	Barley 6 lb./day for 2 days	4.44	Rumenitis
2-744	HAc + straw; pH 4.3; 5 gal./day for 2 days		Normal
2-742	HAc + straw; pH 4.3; 5 gal./day for 2 days		Normal
2-746	NaAc + straw; pH 7.0; 5 gal./day for 2 days		Normal
2-743	NaAc + straw; pH 7.0; 5 gal./day for 2 days		Normal

Experimental rumenitis probably was caused by the products of excessive fermentation of grain. Specific irritants were not identified by experiments conducted to date. Forcible feedings of a solution of acetic acid which had the same pH as was obtained in fermentation failed to cause rumenitis; however, the pH of the introduced acetic acid changed rapidly toward neutrality, while the low pH attained in fermentation was maintained for prolonged periods. It is possible that the prolonged high concentration of hydrogen ions was part or all of the irritant. Solutions of sodium acetate also failed to produce rumenitis.

The cause of spontaneous rumenitis was not proved. Gross and histopathological changes in experimental and spontaneous rumenitis were identical. This identity of

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Fig. 21—Chronic rumenitis of anterior ventral sac. Submucosa contains an epithelial nodule. Leukocytes have infiltrated surrounding connective tissue. Nocht's stain. x 75.

Fig. 22—Foci of hyperkeratosis of dorsal curvature of rumen. Ingesta adheres to hyperkeratotic areas.

Fig. 23—Hyperkeratosis of dorsal curvature of rumen. Epithelium contains excessive layers of hyperkeratinized cells. Nocht's stain. x 100.

Fig. 24—Experimental acute rumenitis of anterior ventral sac. Villi are swollen, hyperemic, and necrotic. Devillation is beginning.

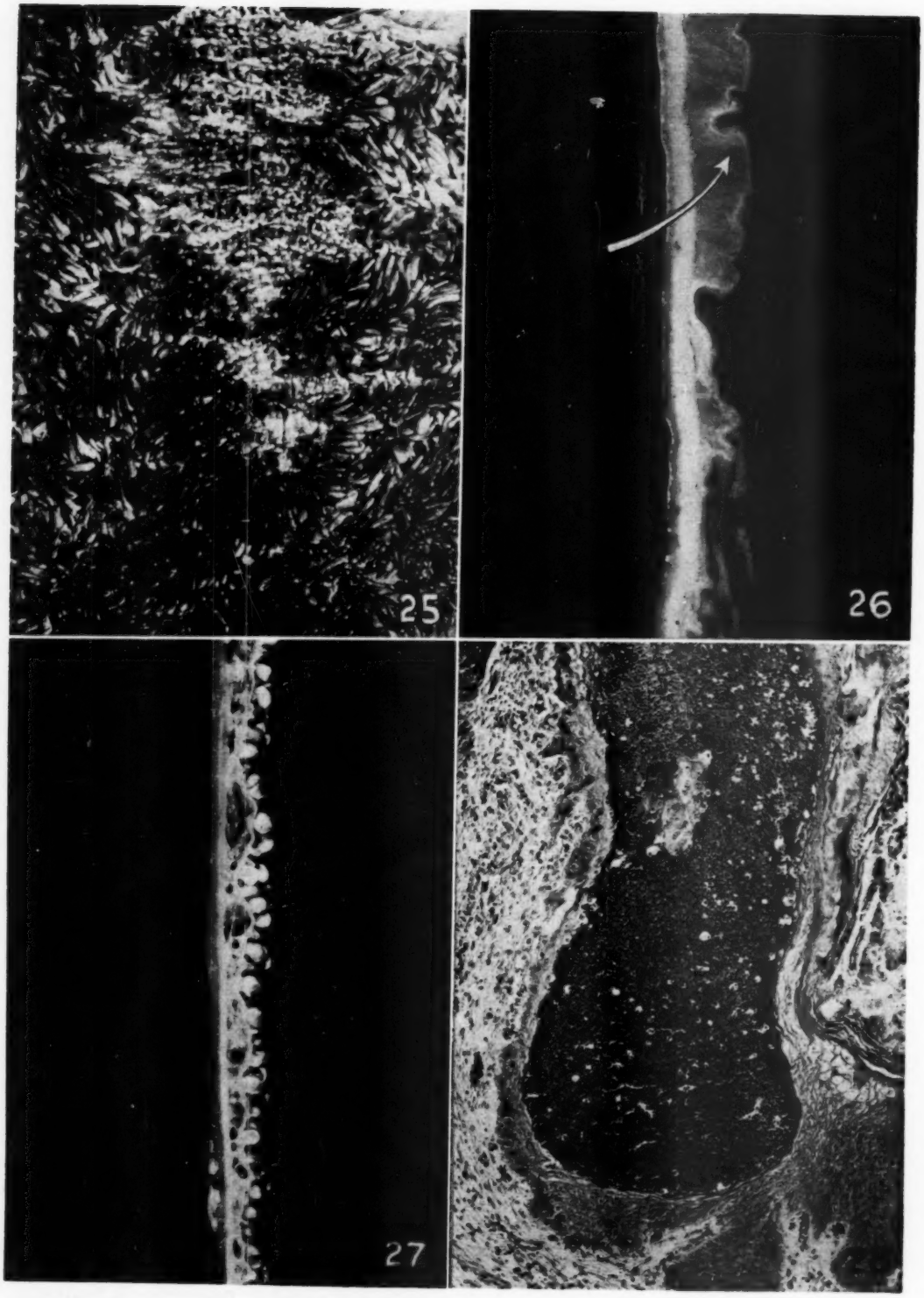
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Fig. 29—Experimental acute rumenitis of anterior ventral sac. Epithelium contains vesicle. Nocht's stain. x 200.

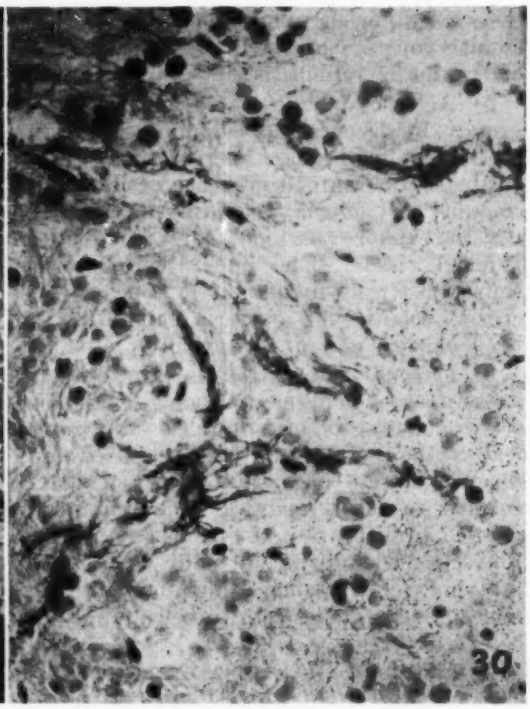

Fig. 30—Experimental acute rumenitis of anterior ventral sac. Submucosa is necrotic and infiltrated with leukocytes and colonies of Spherophorus necrophorus. Nocht's stain. x 400.

lesions indicated possible similarity of causes. Proof of identity awaits further experimentation. The practice in western feedlots of changing feed from roughage to grain in a short period of time may favor reduced ruminal motility and excessive fermentation.

Spherophorus necrophorus is known to be a secondary invader of vesicular lesions in domestic animals. Sheep affected with contagious ecthyma often develop secondary infections of the lips and mouth from *S. necrophorus*. The lesions of foot-and-mouth disease in cattle may develop secondary infection with *S. necrophorus*. In the above mentioned lesions, vesicles form mediums in which *S. necrophorus* can grow and points through which it can penetrate an epithelial membrane. Finding primary vesicular lesions in the rumens of fattened cattle causes the complex of hepatic necrobacillosis to conform more closely with what is known of other types of necrobacillosis: *Spherophorus necrophorus* penetrates an epithelial membrane as a secondary invader.

SUMMARY

1) The stomachs and livers of 1,535 adult fattened cattle were examined. Of

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Fig. 25—Experimental acute rumenitis of anterior dorsal sac. Villi are swollen, hyperemic, and necrotic. Devillation is prominent.

Fig. 26—Experimental acute rumenitis. Cross section of wall of anterior ventral sac, showing edema. Arrow points to edema in submucosa.

Fig. 27—Experimental acute rumenitis. Cross section of wall of anterior ventral sac, showing swelling of villi and gas in submucosa.

Fig. 28—Experimental acute rumenitis of anterior ventral sac. Epithelium contains a large vesicle which is filled with erythrocytes. Nocht's stain. x 100.

these, 37.6 per cent contained lesions of rumenitis and 5.5 per cent contained lesions of traumatic reticulitis.

2) Acute rumenitis was characterized grossly by hyperemia, hemorrhage, necrosis, edema, and gas. Microscopically, the epithelium contained necrosis, vesicles, and colonies of bacteria, including *Spherophorus necrophorus*, which were penetrating injured tissue. The subepithelial tissue showed a strong inflammatory reaction, necrosis, colonies of bacteria, including *S. necrophorus*, gas, and edema.

3) Chronic rumenitis was characterized grossly by depigmentation, devillation, scars, ulcers, pits, and nodules. Microscopically, the epithelium was vesicated and ulcerated; rete pegs were lengthened and formed epithelial nodules. The submucosa showed proliferation of fibrocytes and capillaries and infiltration with eosinophils, lymphocytes, plasma cells, mononuclear macrophages, and multinucleated giant cells.

4) Of the 1,535 cattle examined, 577 showed rumenitis. Of the latter, 91.2 per cent contained lesions in the anterior ventral sac of the rumen.

5) The rumen also showed a high incidence of hyperkeratosis.

6) The association of gastric lesions and liver abscesses showed statistical significance.

7) Stomachs and livers of 630 unfattened calves were examined. The incidence of ruminal lesions and liver abscesses was low.

8) Feeding barley in reasonably large amounts to animals which were not accustomed to grain as a part of their ration, caused the formation of high concentration of acid within the rumen. At autopsy, acute rumenitis was generalized. Grossly and microscopically, the changes were identical to changes observed in spontaneous rumenitis.

9) Feeding acetic acid alone and sodium acetate alone failed to produce rumenitis.

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Course in Electron Microscopy

A course in electron microscopy will be given at Cornell University June 14-26, 1954, by the Department of Engineering Physics. The course is designed for research workers who have recently entered the field of electron microscopy. Address inquiries to Dr. Benjamin M. Seigel, Rockefeller Hall, Cornell University, Ithaca, N. Y.

Photokymographic Studies of Regurgitation and Related Phenomena in the Ruminant

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THE STUDY of the mechanism of regurgitation goes back to the beginning of the nineteenth century. During the years when this aspect of ruminant physiology was under more active investigation, there were details of the process which were not clearly defined. The reason for the discrepancies, in most cases, was that the mechanical lever-type apparatus used for recording the phenomena was inefficient. The negative pressure produced during regurgitation was recorded and measured; however, the inertia of the levers or of the bromoform used in the manometers, and the speed of the action, left some doubt as to whether a true picture of the phenomenon was being obtained. It was decided that photokymographic recordings should be used to increase the efficiency of the recording methods. Mechanical levers were eliminated with the aid of segment capsule manometers.

Toussaint [5] recognized that the outstanding action of rumination was regurgitation and that it was too rapid and complex to study without using a graphic method of registration. He developed the theory that at the moment of regurgitation the glottis closed, dividing the column of air between the nares and the lungs. While the upper free section registered atmospheric pressure, the lower section of the column was influenced by the variations of pressure within the thorax. He recorded an increased negative pressure in the trachea at the moment of regurgitation.

Bergman and Dukes,[1] using methods somewhat similar to those of Toussaint, undertook to test these theories and conclusions by the graphic method. They concluded that the entrance of food into the esophagus is effected by a negative thoracic pressure which was produced by an inspiratory effort with a closed glottis.

Stigler [4] thoroughly investigated the mechanism of rumination in goats. His methods included x-ray investigation. He concluded that the hydrostatic pressure of the rumen contents was sufficient to fill the cardiac portion of the esophagus but that the pressure gradient was not sufficient to produce regurgitation itself. X-ray observations showed that the ingesta were not conveyed into the esophagus by contractions of the stomach. Stigler stated that regurgitation included a sudden inspiratory low pressure followed immediately by an expiratory high pressure. The first phase of the action sucked the ingesta into the esophagus and the second phase aided the propulsion of the fluid mass upwards.

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EXPERIMENTAL APPARATUS

The tracings produced in our investigation employed a photokymographic technique developed especially for this problem. It was decided that in the study the following tracings should be made during regurgitation: (1) intranasal pressure; (2) intratracheal pressure; (3) bolus passage in the esophagus; (4) abdominal movement; (5) jaw movement; and (6) a time tracing. The receiving devices were as follows: (1) For intranasal pressure a glass tube was inserted into the animal's left nostril. (2) The intratracheal pressures were received through a 5-in., 12-gauge needle inserted between the tracheal cartilages halfway down the neck region. (3) the bolus passage in the esophagus was recorded with the aid of a cardiograph tambour. (4) The abdominal movements were received with the aid of a Lombard pneumograph. (5) The jaw movements were received with the aid of a similar pneumograph which was provided with a hook and a spring clamp so that it could be attached quickly to the animal's halter. (6) The time tracing was produced with the aid of a Jacquet graphic chronometer which recorded at intervals of one-fifth of a second. Transmission from the receivers to the recorders was by air through 9 ft. of rubber tubing. The apparatus used for the photokymographic technique of recording consisted of: (1) a series of specially built segment capsule manometers; (2) a variac transformer; (3) a straight filament bulb used as a light source; (4) a photokymograph.

The photokymographic recording manometers were similar to the segment capsule manometers that were described by Wiggers.[6] The over-all length of the capsules was 5½ in. A rubber membrane was stretched over the flattened end of the tube and by adjustments the proper tension was produced. The membrane was held in place by a rubber band wound tightly around the tube. A trapezoid of celluloid was cemented onto the rub-

ber membrane in such a manner that the base of the trapezoid exactly conformed to the flat surface of the tubing. A small, round, concave, first-surface mirror was glued onto the celluloid trapezoid so that the diameter of the mirror was on a chord of the circular end of the tube. The mirror was attached at right angles to the wall of the tube. Six manometers were required and these were arranged in a horizontal row. The light source was a single straight filament projection bulb. The intensity of illumination was regulated by a variac transformer. The light from the projection bulb was restricted to a slit 1 in. wide and 4 in. high, which directed the beam of light to the desired area. The speed of the exposure on the paper of the photokymograph was 12.5 mm./sec.

EXPERIMENTAL PROCEDURES

The equipment was attached to the animal, adjusted, and records were taken when the animal ruminated. A steer with a permanent rumen fistula was used along with 2 normal cows. Throughout the period of rumination, constant adjustments were found necessary to ensure the production of a correct record. If the animal with the fistula refused to ruminate after a period of time, rumination could be stimulated by gently and continually squeezing the ruminoreticular fold. This almost invariably caused regurgitation. The adjustments of the apparatus in the intact animals followed the same general plan. The animals, however, could not be forced to regurgitate. It was, therefore, necessary to attach the apparatus and maintain a quiet environment until the animals regurgitated spontaneously.

The segment capsule manometers were placed so that they were in focus on the lens of the photokymograph. Each of the manometers had an adjustable clamp which allowed vertical focusing of the tube and mirror.

The segment capsules were placed on a table about 3 ft. from the animal and exactly 1 meter from the camera lens. The light source was attached by a clamp to a stand so it could be placed on a level with and as close as possible to the side of the lens of the photokymograph. The variac transformer was placed on a table behind the segment capsules (fig. 1). The light beam was adjusted so it was directed toward all the mirrors of the segment capsules. The filament was single and straight and the focused mirrors reflected an image of it on the lens of the camera (fig. 2).

If all the recorders appeared to be operating correctly, upon regurgitation the motor of the kymograph was started and the lens was opened.

The membranes of the segment capsules had to

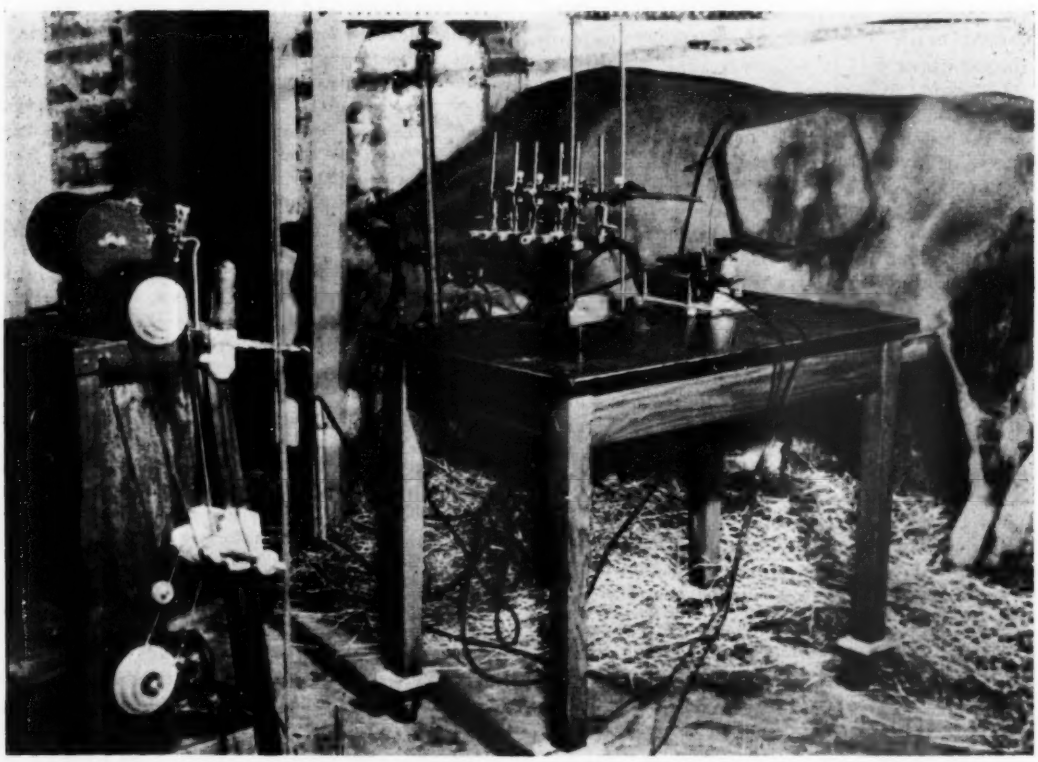


Fig. 1—Illustration showing the recording manometers and receiving devices attached to a Jersey steer with a permanent rumen fistula.

have an inherent natural frequency greater than that which was to be recorded. The majority of the membranes had an inherent natural frequency of 15 to 20 vibrations per second.

The air transmission systems were of equal length; hence, errors due to differences in transmission time were eliminated. The intratracheal pressure manometer was calibrated with the aid of a bromoform manometer.

After the cycles of rumination had been recorded, an attempt was made to correlate the negative pressure of regurgitation with the disappearance of the jugular pulse. The edge of a human carotid pulse cup was covered with strips of adhesive tape. The cup was then cemented over the distended jugular vein with the aid of a liquid adhesive. The adhesive supported the weight of the cup and formed an airtight seal. The cup was then attached to a segment capsule manometer which was focused on the photokymograph.

RESULTS AND DISCUSSION

A series of normal regurgitations were reproduced by the photokymographic method (fig. 3).

The bolus, which had been chewed following a previous regurgitation, was swallowed and was represented in its passage down the esophagus by the break in line II, at AB. The chewing movements ceased immediately before swallowing, and a few positioning movements were made which were represented by the undulations in line III, from K to L. The respirations were represented by the undulations in line IV (nasal).

Line I (intratracheal), and line V (abdominal movements) remained normal until interrupted in early inspiration by a considerable negative pressure. This pressure is outlined during its production by the portion EFG in line I. The tracing of the intranasal pressure showed no negative variation because the glottis was closed. The air from the glottis to the nares was therefore under the influence of atmospheric pressure only. The closure of the glottis was represented by the vibration at H in line IV. A perpendicular line from this point to line I shows that the negative pressure immediately made its appearance. The mass of ingesta which was aspirated produced the undulations IJ, in line II. The mass was squeezed. This action was represented by the movements of the jaws as shown at C and D. This particular curve, and perhaps the one following, does not correspond to the curve of remastication. The fluid was swallowed in most cases in two well-defined portions, which produced the variations in line II, at MN and OP. Immediately after or during the swallow-

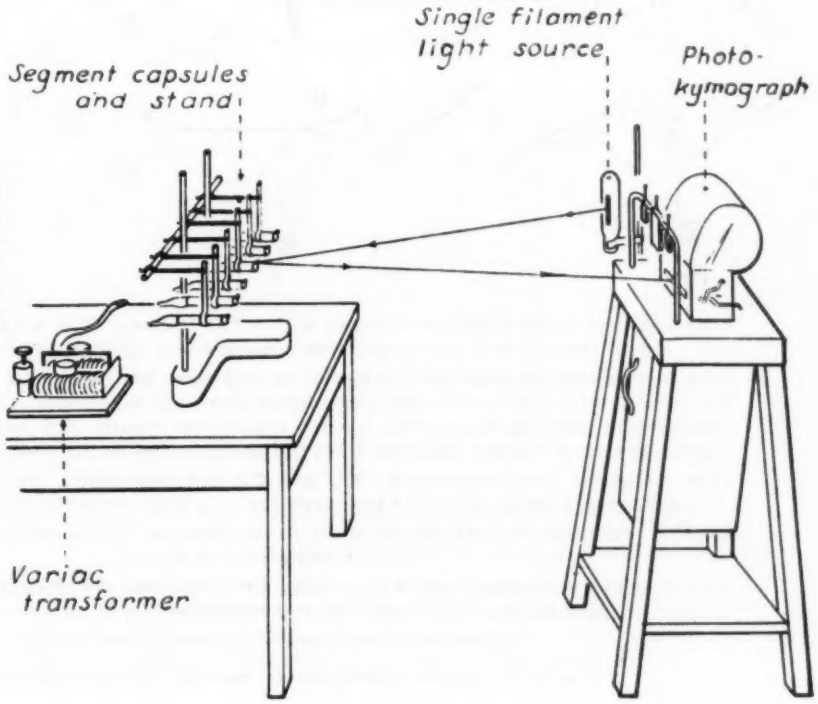


Fig. 2 — Diagram illustrating the arrangement of the light source, the segment capsule manometers, and the photokymograph for recording the process of regurgitation.

ing of the first portion of food, the glottis opened and respirations were resumed (X). The first expiration was, in some cases, more forceful and more prolonged than the others. The chewing movements were resumed as soon as the fluid from the mass was swallowed. The abdominal movements represented by line V showed up during the inspiratory negative pressure. In most cases the record showed a decided dip, QRS, whose depth corresponded to the time of greatest negativity. The abdominal recorder registered the rumen contraction that followed each regurgitation. It is represented by the rise TU.

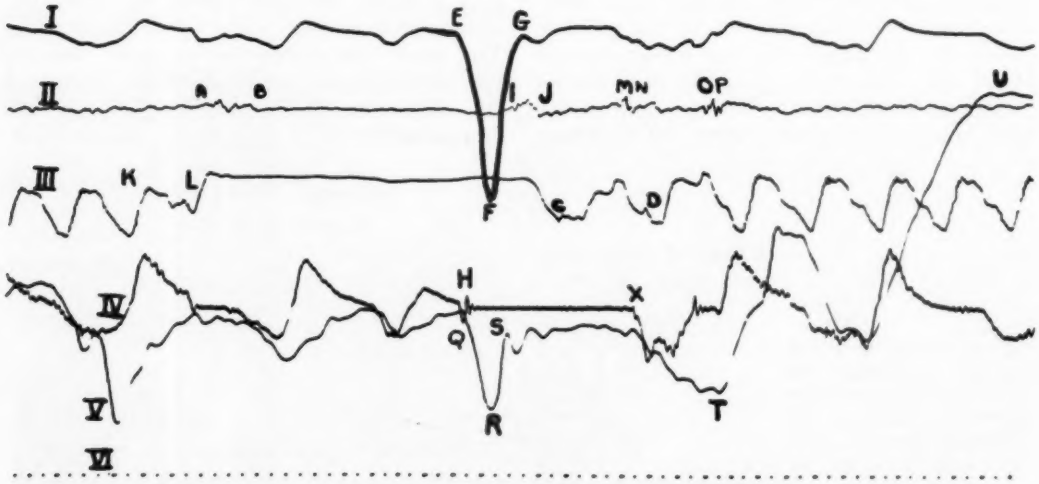
Figure 4 shows a tracing which includes the jugular pulsations. Line I, ABC, represents the intratracheal negative pressure. In this case, it failed to aspirate sufficient ingesta and a second attempt, KLM, was made with a greater negative pressure. The rumen was at the height of a contraction at this time, as shown by the rise, FG, in the abdominal tracing (V). Line II shows a jugular pulse record with the elimination of the pulsation, NO, during the second sustained regurgitation, KLM.

Line III represents jaw movements.

Line IV represents the respirations and point P shows the vibrations caused by the closure of the epiglottis.

Line V represents abdominal movements, and line VI represents the one-fifth-second time record.

From a series of 60 registered, well-defined regurgitations, the following data were compiled: The average time between the swallowing of one bolus and the regurgitation of the second was 3.6 seconds; the interval between the swallowing of the two quantities of fluid which were squeezed from the regurgitated mass was 1.1 seconds; the cessation of nasal pressure variations, which occurs from the closure to the opening of the glottis, represents 3.2 seconds with a variation of 2.0 to 4.0 seconds;



Line I indicates the pressure changes within the trachea. The pressure rises and falls with respirations and is extremely negative at EFG during regurgitation.

Line II indicates the passage of material up and down the esophagus. AB is produced by the passage of a previously masticated bolus down the esophagus. IJ is produced by the regurgitated mass as it proceeds upward toward the mouth. MN and OP are masses of saliva and fluid ingesta squeezed from the bolus in the mouth passing to the stomach.

Line III shows jaw movements. KL and CD are positioning movements previous to swallowing a bolus and after the reception of a fluid mass following regurgitation.

Line IV represents the movement of air in the nostrils. HX is the period of time during which the glottis is closed.

Line V shows abdominal wall movements. QRS indicates the changes at the moment of regurgitation. TU shows the commencement of a rumen contraction.

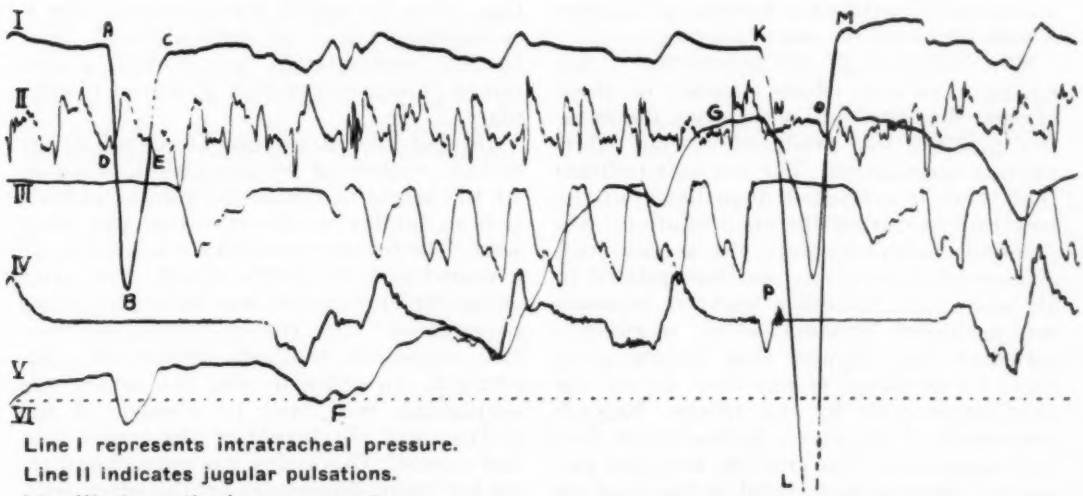
Line VI shows a one-fifth-second time record.

Fig. 3—A normal regurgitation recorded photokymographically.

the pause in the jaw movements represents 3.8 seconds with a variation of 3.6 to 4.6 seconds; and the time required for a tracing to show the beginning of a rumen contraction, following the greatest point of negative pressure in the regurgitation cycle, is 3.2 seconds. From 25 completely recorded rumination cycles, it was found that the average number of jaw movements was 56.4 per bolus. The time required for each jaw movement was 0.05 to 1.2 seconds. The pressure involved in a normal regurgitation compared favorably with the findings of Bergman and Dukes [1] and Stigler.[4] Where a single regurgitation aspirated sufficient ingesta, the intratracheal pressures ranged from 140 to 200 mm. of bromoform. When the ingesta were low in the steer with the fistula, the primary regurgitation was usually normal as to pressure but did not succeed in aspirating sufficient ingesta; a second regurgitation usually occurred within 8.25 seconds. The negative pressure of the second effort invariably exceeded that of the first.

Measurements on the tracings from a mature, intact cow indicated that negative pressures of over 200 mm. of bromoform and approaching 300 mm. of bromoform were involved. Measurements upon an intact heifer showed an average negative pressure of 120 mm. of bromoform during regurgitation. When a second regurgitation was necessary to convey sufficient ingesta to the esophagus, in the steer, it invariably occurred during the contraction of the rumen following the primary attempt. The time of regurgitation was usually early in contraction or at the height of the contraction, apparently to take advantage of the raising of the fluid level during this time. There were times when even a second attempt failed to aspirate sufficient ingesta. In these cases, a third attempt was often made immediately after the second attempt, thus placing it late in the rumen contraction. When a second regurgitation was necessary, the fluid squeezed from the primary regurgitation was in all cases of insufficient quantity to produce a distinct tracing. Further examination of the pressures involved in regurgitation indicated that a positive pressure often preceded and followed the negative pressure. These plateaus were described by Toussaint,[5] and the one following the negative pressure indicated to Stigler [4] that it may have been of some aid in propelling the ingesta up the esophagus.

In the present study there were great



Line I represents intratracheal pressure.
Line II indicates jugular pulsations.
Line III shows the jaw movements.
Line IV represents the passage of air through the nostrils.
Line V indicates abdominal movements.
Line VI shows a one-fifth-second time interval.

Fig. 4—A regurgitation (ABC) that failed to aspirate sufficient ingesta. It is followed by a more forceful regurgitation (KLM) at the height of a rumen contraction (FG). Included in the tracing is a record of the jugular pulsation (II) which disappears with the greater intrathoracic, negative pressure (NO) that occurs during regurgitation.

variations, however, in the amount of positive pressure. Many tracings showed only an amount equal to that produced at the height of a normal expiration and others showed none at all. With mechanical methods of recording there was a question as to whether the positive pressures under consideration were actually present or whether they were placed on the records by the inertia of the lever systems or bromoform manometers. The photokymographic methods show they are present but only as a single plateau indicating a positive pressure which exists until the glottis opens. A pronounced plateau of positive pressure is usually present following a secondary, more forceful regurgitation in the steer with the fistula. None of his records of regurgitation showed the absence of the plateau following regurgitation, and in all cases it was equal to, or slightly greater than, the positive pressure obtained at the height of expiration. A series of tracings were taken while he was being forced to regurgitate by manual pressure. This manipulation indicates that the area most sensitive to this type of stimulation is the ruminoreticular fold, medially toward the esophageal groove. In all cases, the steer attempted to aspirate ingesta by producing a negative pressure; many attempts were unsuccessful, evidently because of incoördinate action of the nerve centers.

Stimulation while the animal was ruminating produced effects similar to those already described. In some cases, the negative pressure was produced without interrupting mastication. The tracings indicate that there is a marked disturbance of the respirations during the manipulation. Abdominal movements were also accentuated. A mass of ingesta was not regurgitated in all cases, even though a negative pressure was produced. Stimulation of the ruminoreticular fold showed that regurgitation could be produced at any time during the rumination cycle by this means. Records were made of the steer's jugular pulse during rumination. The tracings were not primarily intended to be used in the analysis of the jugular pulsation itself but there is enough indication of its presence in most of the tracings to allow an examination of it during regurgitation. The tracings indicate that at the moment of regurgitation there is sufficient negative pressure in the thorax to cause an aspiration of the blood from the jugular veins toward the heart. The increased flow of blood is sufficient in most cases to dampen the jugular pulse during this period. The time required for the negative pressure is approximately one second and the jugular pulse disappears for a similar time.

If the regurgitation is not sustained, the negativity causes little variation in the pulse. The jugular pulse remains visible in many instances when the primary regurgitation effort fails to aspirate sufficient ingesta. In all cases recorded, it disappeared when a more pronounced effort was made upon the second attempt. Records of the pulsations were also made in a quiet, nonruminating animal to show the curves as they would normally appear. A tentative notation was made of the pulse components. A more accurate determination could be made if an electrocardiogram was recorded simultaneously.

Rumen contractions appeared on all of the tracings. The regurgitation of ingesta in all cases initiated the first rumen contraction, which was usually followed by a second. During the forced regurgitations, the rumen contraction following regurgitation was not initiated if the negative pressure occurred at the height of a contraction. If the regurgitation occurred at a time when the rumen was quiescent, then a contraction was in all cases initiated. If a further regurgitation was evoked, a sustained rumen contraction appeared to take place.

During the examination of the numerous records, consistent peculiarities were noted. At the height of the second rumen contraction an inhibition of respiration was often seen. The breath was held for a fraction of a second and the glottis closed. The expiration which followed was invariably more pronounced. The rumen, during contraction, appeared to push against the diaphragm. In order to stop the action, the diaphragm was fixed by closure of the glottis until the height of the contraction had passed. This reflex was noticed and recorded more frequently in the steer with the fistula although there were indications that the same thing occurred in the intact animal. It was also noticed that the bolus manometer was disturbed during this respiratory inhibition, indicating the passage of either saliva or gases. The normal smooth sweep of negative pressure as

shown by the photokymograph was not seen in a few instances. In these cases the regurgitation extended over a greater period of time and although not a forced regurgitation it bore a resemblance to that type of effort.

SUMMARY AND CONCLUSIONS

1) The photokymographic method of recording regurgitation and rumination gives a precise and accurate picture of the pressures involved.

2) The figures compiled from the records of a series of regurgitations indicate that the phenomenon is very precise when normally produced.

3) The negative pressures involved in regurgitation have a wide range depending upon the animal studied. The average pressure in the trachea appears to extend from 120 to 200 mm. of bromoform.

4) The steer with the fistula, when the first regurgitation failed to aspirate sufficient ingesta, often made a second attempt. The second regurgitation invariably occurred at the height of the rumen contraction initiated by the primary regurgitation. This indicated that the animal waited until the ingesta level was sufficiently raised by contraction to ensure the filling of the thoracic esophagus.

5) The records made with other methods failed to give a clear indication of the amount of positive pressure produced immediately before and immediately after regurgitation. Since the manometers show no significant inertia, a more dependable picture of this aspect of regurgitation was obtained by the photokymographic method. The tracings indicate that in most instances the positive pressure is present. The pressures produced in the majority of cases equal those obtained at the height of expiration. This plateau of positive pressure is maintained until the glottis opens.

6) Observations and records made while forcing the animal with the fistula to regurgitate suggest that the stimulation produces a reflex due to a summation of nervous impulses. Regurgitation could be invoked manually at any time during the rumination cycle.

7) Records were obtained during regurgitation showing that the jugular pulse decreases when the negative pressure of regurgitation is sustained and reaches sufficient proportions to significantly increase the flow of blood from the jugular veins toward the heart.

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Phenothiazine in Unweaned Calves

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FOR SEVERAL YEARS, a group of beef cattle have been maintained at the East Texas Pasture Investigation Station of the Texas Agricultural Experiment Station at Lufkin. Originally, the herd was composed primarily of Hereford breeding. Within the last few years, Brahman blood has been introduced. The calves are dropped early in the spring. Together with their mothers, they are maintained on luxuriant fertilized pastures. Rainfall approximates 50 in. annually. At approximately 200 days of age they are weaned, and calves other than those selected for replacement stock are sold for slaughter as milk-fed calves. Internal parasites have never been considered as a source of trouble since eggs-per-gram counts and actual parasites recovered at time of slaughter have been uniformly low.

TABLE 1—Data on Weight Gain of Calves Treated with Phenothiazine

No. of calves	Test started	Test terminated	Ave. start. weight (lb.)	Ave. term. weight (lb.)	Ave. daily gain (lb.)
1951 Test—109 Days					
Control 9	6-1-51	9-18-51	269	471	1.85
Daily 9			274	459	1.70
Periodic 8			306	521	1.97
1952 Test—106 Days					
Control 8	6-1-52	9-15-52	306	536	2.17
Daily 8			306	544	2.25
Periodic 8			307	521	2.02
1953 Test—97 Days					
Control 8	5-7-53	8-12-53	257	442	1.91
Daily 8			257	448	1.97
Periodic 8			257	446	1.95

Since the advent of phenothiazine as an anthelmintic, many studies have been published on its use to control parasitic infestations of farm animals, both as a therapeutic agent in clinically affected animals and as a prophylactic agent to prevent development of clinical parasitism. No attempt will be made to review the voluminous literature on the subject. The practice of feeding phenothiazine free-choice to sheep in salt or mineral mix has received wide acceptance in those areas where sheep are maintained.

Work by Dimock [1] and Todd [2] and others of the Kentucky station emphasize its value in low-level daily doses in the control of strongylid parasites of horses. More recently, the value of a low-level continuous intake for cattle has received the attention of an increasingly large number of workers.[3-6]

In most instances, the cattle were yearlings, or over, or were maintained under conditions where parasitism was a major problem. Few reports were concerned with calves maintained on an adequate plane of nutrition with the incidence of parasitism at a low level.

The study reported herewith was devised primarily to ascertain if there were any noticeable benefits or toxic effects, as determined from daily gain, from phenothiazine administered in daily doses of 1 Gm. and periodic doses of 12½ Gm. per 100 lb. liveweight at 21-day intervals as compared to control calves of the same age, sex, and breed. All calves were unweaned and all were handled exactly alike in every particular. The test periods were respectively 109, 106, and 97 days. Parasitic loads, as determined by regular fecal examinations and by examination of viscera from 2 calves from each experimental group, were uniformly low. The study was conducted over a three-year period.

MATERIALS AND METHODS

Unweaned calves of Hereford and Hereford-Brahman breeding were divided into three groups.

Head, Department of Veterinary Parasitology (Turk); professor, Department of Animal Husbandry (Jones); in charge, East Texas Pastures Investigations Station (Crouch); Texas Agricultural Experiment Station, College Station, Texas.

Grateful acknowledgment is made to Mr. Lee Gibson, foreman, East Texas Pastures Investigation Station, whose coöperation made these observations possible.

TABLE 2—Average Number of Parasites Recovered at Autopsy

	Controls			1 Gm. Daily			12½ Gm. 21-Days		
	H.C.	O.O.	Tr.	H.C.	O.O.	Tr.	H.C.	O.O.	Tr.
1951	206	300	300	54	35	200	10	50	200
1952	525	100	600	60	150	313	260	95	440
1953	460	220	322	280	250	260	1,300	351	280
Ave.	397	207	407	131	145	258	557	165	240

H.C.—*Hemonchus contortus*; O.O.—*Ostertagia ostertagia*; Tr.—Trichostrongylus and Cooperia sp.

Group 1 received no treatment; each animal in group 2 received 1 Gm. of phenothiazine, given in capsule daily throughout the test period; each animal in group 3 received 12½ Gm. of phenothiazine per 100 lb. liveweight at intervals of twenty-one days. The calves were weighed at the start of the test period and each thirty days thereafter until the test was completed.

The average number and kind of parasite recovered at slaughter from 2 animals from each group each year are shown in table 2.

The average daily gain for the three groups for the three-year period was 1.98 lb. per animal for the control groups, 1.97 lb. for the groups receiving 1 Gm. of phenothiazine daily, and 1.98 lb. for the group receiving 12½ Gm. per 100 lb. liveweight each twenty-one days.

Summary

Phenothiazine when given to normal, healthy, unweaned, beef-type calves carrying a light parasite load did not influence daily gain under conditions of this experiment.

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The Teeth of the Ox in Clinical Diagnosis
I. Developmental Anatomy

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THE INCREASING importance of the diagnosis of dental lesions in cattle has led to the research presented in this series of papers. The research was prompted by the almost complete absence of detailed information on normal bovine dental anatomy in veterinary literature. Full understanding of dental pathology must of necessity be based entirely on a prior knowledge of normalcy. We can not recognize the abnormal unless we can first recognize the normal.

In preparation, 7,520 average, normal animals were studied in slaughterhouses as follows: Toppenish and Ellensburg, Wash., 40; Phoenix, Ariz., 155; Los Angeles, Calif., 3,166; East St. Louis, Ill., 4,159.

Because these animals were in excellent condition in almost all cases, being primarily young, finished beef animals (over 5,000 had not yet completed eruption of the permanent teeth), they offered an unexcelled medium for studying normal dental anatomy.

In addition, many herds of normal animals were examined; 2,140 live animals suspected of having dental fluorosis were given oral examinations. Sections of the head of the fetal calf were prepared for the purpose of studying the developing tooth buds.

This research was conducted primarily by visual examinations. Dissections were made of both normal and abnormal conditions. Gross examinations were supplemented by the use of a low-power magnifying glass, a Leitz dissecting microscope, and conventional microscopes, using both reflected and transmitted light. Conditions of pathology were photographed, then dissected, ground or fractured, and studied under varying magnifications. No attempt was made to demonstrate bacteria in cases of dental caries, nor were any identified.

The studies of developmental anatomy* were made on embryos and fetuses obtained at the abattoir from healthy female animals at time of slaughter. Those tissues which were prepared for microscopic sections were chosen from the very young fetus because bone formation had not developed to a point where it could interfere with conventional embedding and cutting procedures.

With the exception of three, all photographs in this series were taken by the author. Figures 1 and 2 were taken at the photography laboratory at the Medical College of the State of South Carolina from histological sections prepared by the staff of the tumor clinic of the University of Southern California from material furnished by the author.

Because true color definition can not be obtained in black and white photography, illustrations in full color would have been better. However, due to the prohibitive cost of color plates for publication, this was not possible. Where important, a color interpretation, made by referring to reproductions in color, has been given in the descriptions of the illustrations. In many cases, the portrayal of average pathology was impossible due to the limitations of photography already mentioned. Therefore, the illustrations are simply an adjunct to the more important substance of the text.

The bibliography appended hereto was used as a guide to chronology and as a laboratory manual of procedure. Anatomic structures herein reported were demon-

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Grateful acknowledgment is made to farmers, packing-plant managements and personnel, veterinary inspectors of the U. S. Bureau of Animal Industry and of the State of Washington, to members of the staff of the Dental College and Tumor Clinic at the University of Southern California, to members of the staff of the Medical College of the State of South Carolina, to Dr. Isaac Schour, director of graduate studies at the University of Illinois Dental School, and to Dr. Harry Sicher, Department of Anatomy of the Dental College, Loyola University, Chicago, all of whom, by their coöperation and helpful suggestions, have helped make this study possible.

* In comparative dental anatomy, the ox is considered to have but three pairs of mandibular incisor teeth. The "fourth incisors" are truly modified canine teeth. However, morphologically, and because of common veterinary usage, the anterior teeth of the mandible will be grouped together as incisors.

strated to the satisfaction of the author in all cases unless reference is given to substantiate the observation.

It is the intention of the author to confine reported pathology to his own observations, realizing the limited scope, and realizing also that this article is not all-inclusive. The material is elementary and subject to revision with further developments in the knowledge of oral anatomy and pathology.

DEVELOPMENTAL ANATOMY

Before the clinician can understand gross anatomy and pathology of teeth, a knowledge of embryology and histology is necessary. Following is a brief resumé of these subjects, confining the scope to the dental and periodontal tissues.

The tooth proper is composed of four tissues, three calcified and one soft.

1) Enamel is the hard outer covering of the anatomical crown of the tooth. It is epithelial in origin.

2) Dentine makes up the main bulk of the tooth, underlying the enamel of the crown, and giving form and structure to the root. It is mesodermal in origin.

3) Cementum is the calcified outer covering of the root of the tooth. It is also found in the infundibula. It is mesodermal in origin.

4) Pulp is the soft tissue within the central cavity of the dentinal portion of the tooth. It is composed of connective and dentine-forming tissues, intercellular substances, vessels, nerves, all of mesodermal origin. It communicates with the periodontal membranes through the apical foramen at the apex of the tooth root.

The teeth are rendered functional by their implantation in the dental arch. The supporting membranes are four in number.

1) Cementum gives firm attachment to the fibers of the periodontal membranes.

2) Periodontal membrane is largely made up of tough, fibrous connective tissue and is firmly attached to cementum, alveolar bone, and gingiva.

3) Alveolar bone forms the socket to which the ligamentous fibers of the periodontal membrane are attached. In the ox, the sockets are relatively shallow in the case of the incisor teeth and allow them considerable movement.

4) Gingiva is the fibrous tissue, covered by modified mucous membrane which covers the alveolar processes of the jaws and surrounds the neck of the teeth, giving further attachment thereto.

Dental Proliferation.—Early in embryonic life, there arises an epithelial thickening near the free margin of the jaws—the dental lamina. It is composed of superficial squamous cells and a basal layer of columnar cells. It is separated from the mesoderm by a basement membrane. The dental lamina proliferates at various intervals at points corresponding to the future deciduous teeth and permanent molar teeth (fig. 1). The buds thus formed invade the underlying mesoderm and become invaginated in the form of a cap to enclose a condensation in the mesoderm, the primitive dental papilla, which is an area of specialization of the mesoderm (fig. 2). Formation of the dental papilla is initiated and stimulated by the presence of the dental lamina. The enamel organ is derived from the dental lamina, while the dentine and pulp are derived from the dental papilla. With further proliferation and increased depth of the concave surface, the enamel organ gradually passes from the cap stage to the bell stage.

Cell Differentiation.—At the time of the bell stage of the enamel organ, final differentiation of its cells has been reached. They are four in number.

1) The tall columnar inner enamel epithelium which lines the invaginated concavity of the enamel organ (these cells no longer show mitotic figures but are differentiated into the enamel-forming cells—ameloblasts);

2) the low squamous cells of the stratum intermedium which overlie the ameloblasts;

3) the star-shaped cells of the stellate reticulum which is enclosed by the outer enamel epithelium and makes up the bulk of the enamel organ at this stage;

4) the flat outer enamel epithelium which lines the convex surface of the enamel organ.

During the differentiating bell stage of the enamel organ, the outer cells of the dental papilla, which face the inner enamel epithelium, become larger and assume a columnar form. These are the dentine-forming cells—odontoblasts.

As the dental papilla and the enamel organ take on their full form, differentiation of the surrounding mesoderm overlying the enamel organ occurs. Fibrous tissue forms around the outer surface of the enamel organ and the base of the dental papilla. This is the beginning of the dental sac which will give rise to the cementoblasts, fibroblasts, and osteoblasts from which most of the supporting structures of the tooth are formed. The dental sac severs the connection of the enamel organ with the oral epithelium.

The dental lamina continues to extend deeper and lingually from the bud of the deciduous tooth. It develops into the enamel organ for the corresponding permanent tooth and passes through the same phases of development as do the deciduous teeth. The permanent molar teeth, having no corresponding deciduous teeth, arise directly from the dental lamina. Therefore, histogenetically the permanent molar teeth belong to the deciduous dentition.

Functions of the Enamel Organ.—The primary functions of the enamel organ are listed.

1) It determines the pattern, shape, and size of the future crown of the tooth.

2) Its inner layer, the ameloblasts, are responsible for enamel formation.

3) It stimulates the mesoderm to form the dentine of the crown.

4) It gives rise to an epithelial sheath which guides the shape of the forming root.

5) It produces the enamel cuticle, the outer layer of the future anatomical crown.

6) It provides the epithelial attachment at the gingival level after eruption of the tooth.

Amelogenesis.—The formation of enamel begins with a reduction in the stellate reticulum. The outer enamel epithelium unites with the stratum intermedium beginning at the incisal margin in the case of incisor teeth, or at the tips of the cusps in the case of the cheek teeth. Connective tissue papillae containing capillary loops project into the outer enamel epithelium. A basement membrane forms between ameloblasts and odontoblasts.

The dentino-enamel junction at this point shows the corrugated contour of the vertical striations of the future tooth. This corrugated contour is evidenced by the vertical grooves which are present on all newly formed permanent incisor teeth in the ox, as well as the cheek teeth. The corrugated contour gives additional strength to the tooth, helping to prevent cleavage of enamel from dentine in the mature tooth when subjected to traumatic influences.

The ameloblasts, as mentioned before, show no mitotic activity. They are hexagonal in cross section, and nuclei are located in the pole away from the dentino-enamel junction. These cells are connected to each other and to the stratum intermedium by fine intercellular bridges. The dentinal ends of the ameloblasts are held together by a honeycomb network of intercellular bars which are a condensation of the intercellular substance between the ameloblasts.[1]

Amelogenesis takes place in two distinct and well-defined phases. In the incisors of the ox, apposition, the first phase, is largely completed before maturation, the second phase, begins. The appositional phase consists of the forming of the enamel rods and the primary deposition of inorganic salts up to thirty per cent of their final concentration. When apposition is complete, the enamel has the consistency of a jelly-like mass with all of the structural elements complete with the exception of the deposition of the remaining inorganic salts. Maturation consists of the final calcification and hardening of the enamel.

Amelogenesis lags behind dentinogenesis in the crown of the tooth, though following closely. Calcification of the dentine is well advanced before the ultimate calcification of amelogenesis begins.

Appositional Phase of Amelogenesis.—Apposition begins with the deposition, by the ameloblasts, of a hexagonal prism of protoplasm on the basement membrane at the dentino-enamel junction. The process is initiated at the tip of the incisal margin on the incisor teeth and at the tips of the cusps of the cheek teeth. The direction of the prism is at an angle to the basement membrane, toward the eventual functional incisal margin or occlusal surface of the completed tooth. The deposition of additions to the enamel prism continues in rhythm until the ultimate length of the prism is attained. The interprismatic substance is formed concurrently and is an extension of the terminal intercellular bars described previously. Adjacent ameloblasts toward the root of the tooth begin amelogenesis in graded intervals giving a horizontal pattern to the process. This is termed the incremental pattern. Thus, the prisms at the tip of the tooth will have attained their full length at a time when apposition is just beginning at the middle of the tooth. This fact is important in explaining the pattern of hypoplastic defects which involve only a portion of the completed enamel. The time of formation of any given incremental layer of enamel is in direct proportion to its distance from the growth center on the dentino-enamel junction at the tip of the tooth.

Primary calcification continues concurrently with prism formation during the

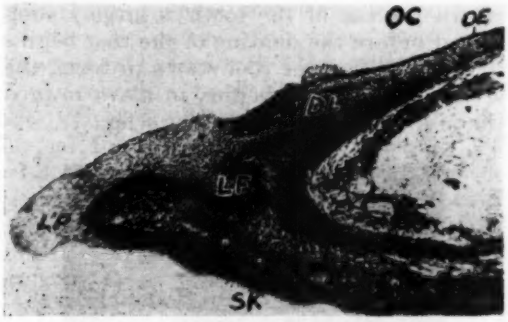

Fig 1—Photomicrograph of a sagittal section of the free tip of the lower jaw of a 2-month fetal calf. OC—oral cavity; OE—oral epithelium; DL—dental lamina forming tooth bud of incisor; LF—lip furrow; LP—tip of the lip; SK—skin.

appositional phase of amelogenesis. The amount of inorganic salts deposited at this time amounts to approximately one third of the ultimate inorganic salts of the completed tooth.[2] This amount of calcification is not sufficient, however, to give structural hardness to the developing enamel.

Maturational Phase of Amelogenesis.—Maturation of the enamel consists of the impregnation of the organic matrix of the enamel with inorganic salts to a point where the final enamel structure is 96 to 98 per cent inorganic salts and the rest organic material and water. The interprismatic substance is calcified at a slower rate and to a slightly less degree than the enamel prisms.

Maturation starts at the highest tips of the cusps of the cheek teeth and at the lateral portion of the incisal margin of the incisor teeth. It continues toward the root in a horizontal pattern, the salts penetrating the entire thickness of the enamel as they go. Therefore, it is apparent that maturation does not follow the incremental pattern of prism formation but follows the pattern of eruption. Maturation is not quite complete at the time of eruption, that portion near the root finishing shortly thereafter. The fact that the entire thickness of the enamel is being calcified on any particular horizontal level gives a basis for differentiating defects of hypocalcification from defects of hypoplasia in the completed tooth.

The Fate of the Ameloblasts Cuticle Formation.—When the enamel prisms have reached their ultimate length, the ameloblasts secrete the enamel cuticle. This is composed of two layers, the primary calcified inner layer overlying the tips of the enamel prisms and the secondary hornified layer overlying the first. When eruption of the tooth occurs, the redneed enamel epithelium unites with the oral epithelium and then gradually peels off of the crown as the tooth comes into use.

Properties of Completed Enamel.—The completed enamel differs from other calcified animal tissues in many aspects:

1) It is the hardest tissue of the body and highest in inorganic salts.

2) It is the only calcified tissue arising from epithelium.

3) It varies in formation and structural elements.

4) It contains no living tissue which can be identified as such.

5) It has no known power of regeneration.

6) It is changed only by the chemical and physical action of its environment.

7) The uninvested portion is not subject to resorption.

8) It has no sensory apparatus.

9) It depends on the contained moisture to maintain structural soundness. Without moisture, it becomes brittle and easily fractured.

10) The fluids within the enamel communicate by diffusion with the fluids of the mouth.

Enamel, as it forms, records within its form and composition its own stages of formation, calcification, and reactions to systemic disturbances.[3] This record is permanent. Following the completion of enamel formation, the enamel no longer has the recording properties but reflects only external environmental influences.

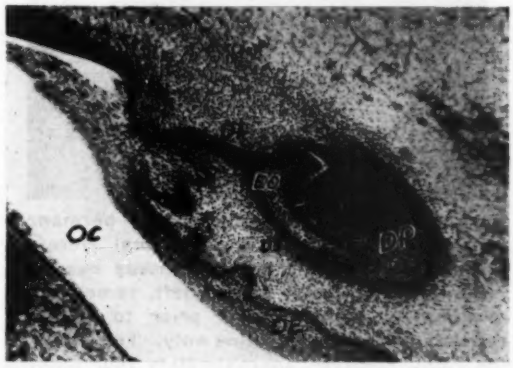

Fig. 2—Photomicrograph of a sagittal section through the center of a developing tooth bud in the maxilla of a 2-month fetal calf. OC—oral cavity; DL—dental lamina; EO—enamel organ; DS—dental sac; DP—dental papilla; OE—oral epithelium.

Dentinogenesis.—The subject of dentine formation will be dealt with only briefly since a detailed description of the process will not serve to amplify to any great extent the visual diagnosis of dental pathology as encountered grossly in the ox.

Dentinal Matrix Formation.—During the bell stage of the enamel organ, the peripheral cells of the dental papilla become tall columnar cells which arrange themselves opposite the ameloblasts. Dentinogenesis precedes amelogenesis slightly, starting at the growth centers on the dentinoenamel junction at the tips of the teeth, and proceeding toward the root in successive horizontal waves. The pattern of any given incremental wave is that of a modified cone with the apex toward the incisal margin or tip of the cusp as the case may be. The incisors, therefore, have but one growth center, while the cheek teeth have one growth center for each cusp presented.

Dentine formation takes place from without inward. Dentine is apparently formed by the action of the odontoblasts on intercellular Korff fibers.[4] As the dentine forms and the odontoblasts recede, a cytoplasmic process is formed and left embedded in the dentinal matrix. This fiber maintains its attachment to the odontoblast and is thought to be instrumental in transmitting nervous stimuli to the nerve fibers of the pulp.[5]

As dentine formation goes on, the pulp necessarily recedes to make way for it. The crown portion of the tooth is largely completed before the dentine of the root begins to form. When the root starts to form, the crown of the tooth begins to move toward the site of eventual eruption (fig. 3). As the crown moves, the root is formed behind under the guidance of the epithelial sheath which is an extension of the enamel organ. Dentine formation in the root proceeds from without inwardly, starting at the epithelial sheath and proceeding toward the apex in a rhythmical, horizontal, incremental pattern resembling a cone, with the apex toward the crown.

Fig. 3—Incompletely formed third permanent incisor teeth removed from normal Hereford steers. Incompletely calcified tissue has been removed. The tooth on the left, removed approximately eight months prior to eruption, consists of calcified dentine only. The tooth on the right, removed approximately four months prior to eruption, consists both of calcified dentine and enamel. The root on the latter has begun to form, the crown portion having started to move out of the bony crypt toward the site of eruption.

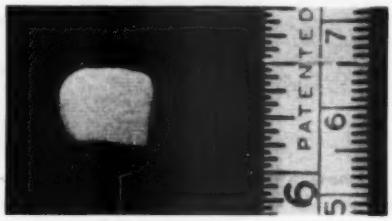

Fig. 4—Incompletely formed second permanent incisor tooth removed from a normal Hereford steer approximately nine months prior to eruption. Incompletely calcified tissue has been removed. This demonstrates the horizontal incremental pattern of dentinal calcification.

Calcification of Dentine.—Calcification of the dentine follows very closely the secretion of dentinal matrix, and follows the incremental pattern in time and rhythmicity. Thus, dentinal calcification is well-advanced before maturation of the enamel matrix gets started (fig. 4).

Properties of Completed Dentine.—Once formed, dentine is not subject to calcium withdrawal but can be resorbed under certain conditions by the action of osteoclasts. Dentine reflects systemic disturbances as well as disturbances in calcium metabolism by variations in structure during the formative period. The variations in dentine are not as easily detected on gross examination as are variations in enamel formation. Normal dentine is made up of from 61 to 73 per cent inorganic matter and the rest is organic matter and water.[6]

Secondary Dentine.—After the tooth has erupted, has become functional, and is subjected to the stresses of normal attrition, the odontoblasts continue to deposit dentine which is inferior in quality to primary dentine. This is the secondary dentine. It

serves the purpose of preventing exposure of the pulp as the tooth wears away. The process of secondary dentine formation may continue until the primary pulpal portion of the tooth is obliterated and the apical foramen is apparently closed. The closure of the apical foramen may be observed in the aged subject when the tooth has worn well past the cemento-enamel junction and only a portion of the root remains.

Sensitivity of Dentine.—As in the human being, dentine exposed in the normal process of attrition is not sensitive on the surface. The processes of sensitivity recede with the formation of secondary dentine.

Functions of the Dentine.—The dentine furnishes the bulk of the supportive portion of the tooth and gives a firm elastic base to the brittle enamel which could not endure without it. Its surface is corrugated to strengthen the support of the enamel. In addition, it provides much of the masticatory surfaces of the cheek teeth. Because of its softer relative structure, as compared to enamel, it wears away at a faster rate, thus preserving the ridges of enamel and materially aiding in the comminution of food. In the incisor, it makes up much of the useful surface of the tooth in the older subject.

SUMMARY

The essentials of dental embryology and histology have been covered briefly. Included are explanations of enamel formation and calcification, dentine and secondary dentine formation, and the functions and properties of each. A thorough understanding of the information presented will provide the clinician with a primary stone in the foundation for rational diagnoses of dental irregularities in contrast to normal dental anatomy.

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[1] Noyes, B. N., Schour, I., and Noyes, H. J.: Oral Histology and Embryology, Lea and Febiger, Philadelphia, (1948): 75-78.
[2] *Ibid.*: 82.
[3] *Ibid.*: 111.
[4] *Ibid.*: 117.
[5] *Ibid.*: 133.
[6] *Ibid.*: 124.

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The Controlled Feeding of Fluorine, as Sodium Fluoride, to Dairy Cattle

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In 1949, Stanford Research Institute undertook a study to determine the effect on cattle of fluoric effluents emitted to the atmosphere by a large industrial plant. At the outset, it was apparent that two aspects of the problem urgently needed solution. These can be summarized briefly as follows:

1) What is the tolerance of cattle to soluble fluorides,* *i.e.*, what amounts of fluoride can be ingested by a cow over extended periods without harm?

2) What are the pathognomonic symptoms of fluorosis in cattle?

An extensive and critical review of the literature [21] indicated that the reliable and unique symptoms of fluorosis in cattle are mottled, stained, and worn incisors; bilateral lameness; and exostoses of the long bones, jawbone, and ribs. In addition, many secondary, but not unique, symptoms are often noticed.

The literature study also suggested that a daily ingestion of 1 mg. of soluble fluorine per kilogram of body weight represented the safe border-line level for ingestion over extended periods.

Although use was made of the recent studies on small animals [5–11, 17, 22–26] and of the feeding experiments with sheep and cattle,[1–4, 7, 11–13, 16, 18, 20, 27] no clear-cut data were available on the effect of feeding soluble fluoride-fluorine. Unfortunately, most of the experiments on large animals reported in the literature concerned the feeding of relatively insoluble fluorides (as rock phosphate). The relationship between toxicities of soluble and less soluble fluorides has been based upon data from the experiments on small animals, with the attendant uncertainties in translating results into terms applicable to ruminants.

In order to extend our knowledge, it was necessary to find an area where cattle had a natural exposure through drinking water high in fluorides as their only source of fluorine. Such fluorides, being in solution, could be expected to have maximum toxicity. Such an area was found and studies undertaken north of Phoenix, Ariz.[19] The results provided striking confirmation for the conclusion suggested by the literature, *i.e.*, that the safe border-line level of ingestion of soluble fluorides for cattle is about 1 mg. of fluorine per kilogram of body weight per day.

Since there was a paucity of data on long-term feeding of soluble fluorides, a controlled feeding experiment was started in December, 1949. Dairy cattle fed fluorine as sodium fluoride are being closely watched, examined periodically, and compared with control cattle over a period of five to six years. This paper is in the nature of a progress report at the half-way point in the experiment. The objective of this experiment was to determine the safe

TABLE 1 — Milk and Butterfat Production in the First Lactation of Holstein-Friesian Heifers Fed Sodium Fluoride

Cow (No.)	Total fluorine in diet (mg./kg. body wt./day)	Days in milk	Total milk (lb.)	Total fat (lb.)	Ave. milk per day (lb.)	Ave. fat per day (lb.)
1	0.15 to 0.3	305	11,801	450	38.7	1.48
2	0.15 to 0.3	305	7,253	268	23.8	0.88
3	0.15 to 0.3	305	9,601	378	31.5	1.24
4	0.15 to 0.3	305	13,110	516	43.0	1.69
Ave., group 1			10,441	403	34.3	1.32
5	1.0	305	11,511	389	37.8	1.28
6	1.0	305	11,369	401	37.3	1.31
7	1.0	305	11,553	384	37.9	1.26
8	1.0	305	10,003	387	32.8	1.27
Ave., group 2			11,109	390	36.5	1.28
9	1.5	305	11,058	443	36.2	1.45
10	1.5	305	11,899	428	39.0	1.40
11	1.5	305	11,503	399	37.7	1.31
12	1.5	305	9,852	314	32.3	1.03
Ave., group 3			11,078	396	36.3	1.30
13	2.0	305	12,448	435	40.8	1.43
14	2.0	305	11,330	443	37.2	1.45
16	2.0	305	12,326	413	40.4	1.35
Ave., group 4			12,035	430	39.5	1.41
17	2.5	305	12,116	416	39.7	1.36
18	2.5	305	9,741	347	31.9	1.14
19	2.5	305	11,246	359	36.9	1.18
20	2.5	305	12,758	386	41.8	1.27
Ave., group 5			11,465	377	37.6	1.24

From the Stanford Research Institute, Stanford, Calif.

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* The element fluorine, because of its extreme chemical activity, never occurs in the free state in nature but rather in combination with other elements. Compounds containing fluorine are commonly called fluorides. Therefore, in this paper the word "fluorine" refers to the fluorine content of the particular fluoride in question.

levels of fluorine which could be fed to cattle under controlled experimental conditions. Although conclusions must await the end of the experiment, the information already gleaned will be of interest.

EXPERIMENTAL DATA

In December, 1949, 20 bred Holstein-Friesian heifers, 2 years old, were purchased from herds free of tuberculosis and brucellosis and brought to the Lavacre Holstein Farm, Modesto, Calif. Ten additional heifers were chosen from the Lavacre Farm herd. They were selected on the basis of uniform potential production ability, time of calving, and weight. They were placed in a drylot, fed alfalfa, and studied for ten days. Finally, 20 were selected and divided into five groups of 4 each.

During December, 1949, and January, 1950, they were broken to farm routine. Still photographs of the animals, colored motion pictures showing them walking, and detailed pictures of their incisor teeth were taken. This photographic procedure has been repeated at six-month intervals. Initially, the condition of the incisor teeth was recorded by a single frontal photograph. As the fourth incisor teeth erupted and began to show changes, it was necessary to photograph the right and left incisor teeth separately.

During the latter part of December, 1949, a commercial grain mixture containing 17 per cent protein was added to the diet and fed at a level of 3 lb. per head per day. When the sodium fluoride feeding was begun in February, 1950, the grain was increased to an average of 6 lb. per head per day. The grain mixture was varied with the stage of lactation, condition of the animal, et cetera. Throughout the three-year period, each cow has received an average of 7 lb. of grain mixture per day. The animals were maintained on irrigated pasture and brought to the barn morning and evening when the hay and grain mixture was fed. The pasture grass was composed of Ladino clover, rye, blue, Bermuda, and Dallis grasses.

TABLE 2—Milk and Butterfat Production in the Second Lactation of Holstein-Friesian Heifers Fed Sodium Fluoride

Cow (No.)	Total fluorine in diet (mg./kg. body wt./day)	Days in milk	Total milk (lb.)	Total fat (lb.)	Ave. milk per day (lb.)	Ave. fat per day (lb.)
1	0.15 to 0.3	305	13,426	538	44.0	1.76
2	0.15 to 0.3	239	5,865	221	24.1	0.92
3	0.15 to 0.3	305	10,718	460	35.1	1.51
4	0.15 to 0.3	305	10,492	442	34.4	1.45
Ave., group 1			10,125	415	34.4	1.41
5	1.0	305	12,078	438	39.6	1.44
6	1.0	305	12,078	438	39.6	1.44
7	1.0	305	11,074	403	36.3	1.32
8	1.0	303	8,373	337	27.6	1.11
Ave., group 2			10,901	404	35.8	1.33
9	1.5	305	11,531	452	37.8	1.48
10	1.5	305	13,178	467	43.2	1.52
11	1.5	305	12,309	441	40.3	1.44
12	1.5	305	12,990	556	42.6	1.82
Ave., group 3			12,502	479	41.0	1.57
13	2.0	305	12,988	473	42.5	1.55
14	2.0	305	13,112	528	42.9	1.73
16	2.0	305	11,568	420	37.9	1.37
Ave., group 4			12,556	474	41.1	1.55
17	2.5	305	12,935	497	42.4	1.63
18	2.5	305	11,335	414	37.1	1.36
19	2.5	305	10,796	371	35.3	1.21
20	2.5	305	12,712	405	41.6	1.32
Ave., group 5			11,945	422	39.1	1.38

TABLE 3—Group Averages and Standard Deviations of Milk and Butterfat Production in the First and Second Lactations of Holstein-Friesian Heifers Fed Sodium Fluoride

First lactation	Total fluorine in diet (mg./kg. body wt./day)	Average milk per day (lb.)	Average fat per day (lb.)
Group 1	0.15 to 0.3	34.3±8.4	1.32±0.35
Group 2	1.0	36.5±2.5	1.28±0.03
Group 3	1.5	36.3±2.9	1.30±0.19
Group 4	2.0	39.5±1.9	1.41±0.05
Group 5	2.5	37.6±3.9	1.24±0.10
Herd average		36.8±1.9	1.31±0.06
Second lactation			
Group 1	0.15 to 0.3	34.4±8.0	1.41±0.35
Group 2	1.0	35.8±5.7	1.33±0.16
Group 3	1.5	41.0±2.5	1.57±0.17
Group 4	2.0	41.1±2.8	1.55±0.03
Group 5	2.5	39.1±3.4	1.38±0.18
Herd average		38.3±3.1	1.45±0.11

The feedstuffs (pasture grass, grain mixture, and alfalfa hay) and the water consumed by the cattle were analyzed for their fluorine content at the beginning of the experiment and at irregular intervals thereafter, and whenever there was a change in the source of feeds. On a dry basis, these materials have been found to contain fluorine as fluoride in the following amounts: pasture grass, 3 to 11 parts per million (p.p.m.); alfalfa hay, 3 to 5 p.p.m.; commercial grain mixture, 5 to 20 p.p.m. Well water has consistently contained a low fluorine level of 0.1 to 0.2 p.p.m.

Knowledge of the grain mixture and hay intake and calculations of the water [6] and green pasture [14] consumption showed that each cow consumed approximately 0.15 to 0.3 mg. of natural fluoride per kilogram of body weight per day. Each experimental cow received, in addition, the calculated amount of fluorine in the form of sodium fluoride (analytical reagent grade), added dry to the moist grain mixture at the time of the morning feeding. The fluoride supplement was recalculated for each cow at three-month intervals on the basis of body weight and food consumption. The total amount of fluorine the animals received was as follows, measured in milligrams of fluorine per kilogram of body weight per day.

Group 1 (control)	cows 1 to 4	0.15 to 0.3 mg.
Group 2	cows 5 to 8	1.0
Group 3	cows 9 to 12	1.5
Group 4	cows 13 to 16	2.0
Group 5	cows 17 to 20	2.5

Cattle weights were obtained at three-month intervals, with teeth and body examinations carried out monthly. Urine samples, obtained for determination of fluoride excretion, were taken at three- to four-month intervals. They were taken in the morning, after each cow had been placed in her stanchion, and were then shipped to the Stanford Research Institute for fluorine analysis. Milk and butterfat production records were checked monthly under the supervision of the Dairy Herd Improvement Association (D.H.I.A.).

The experiment was designed to determine between the damaging and nondamaging boundary levels of fluorine when fed in the soluble form as sodium fluoride. It was devised to test the influence of various levels of soluble fluoride on dairy cows over long periods and, particularly, its effect on *(a)* lactation; *(b)* weight; *(c)* teeth; *(d)* general health; *(e)* bones and tissues.

RESULTS AND DISCUSSION

Effect on Lactation.—A summary of the D.H.I.A. production records of the individual cows for the first and second lactations is presented in tables 1 and 2. Group averages and the standard deviation during each of the 305-day test periods are shown in table 3. Statistical analyses of the data were carried out by applications of the "t test" and "analysis of variance." Inspection of the data shows that there were no significant differences in the milk or butterfat production of the controls or of the animals fed the various amounts of sodium fluoride. There were, however, greater variations in milk and fat yields within each group during the second lactation than during the first. In no case is the average for the control group significantly different from the average for any experimental group receiving sodium fluoride supplements.

Effect on Weight.—Fluorine, when fed in the form of sodium fluoride at the levels used in this experiment, exerted no detrimental effect upon weight during the first thirty-six months of the experiment. Table 4 presents the weights of the individual cows and the group averages during this period. Since the cows were 2 years old when the experiment started, the critical growth period had already passed. Although the cows had been bred when the sodium fluoride feeding began, no favorable weight difference has developed in the group fed no fluoride. At the end of three years, there was a spread of only 100 lb. between the average weights of the lightest and heaviest groups.

TABLE 4—Average and Individual Body Weights of Cows Fed Sodium Fluoride

Cow (No.)	Total fluorine in diet (mg./kg. body wt./day)	1/5/50 Group (ave.)	1/13/51 Group (ave.)	1/10/52 Group (ave.)	3/10/52 Group (ave.)	7/8/52 Group (ave.)	10/15/52 Group (ave.)	1/13/53 Group (ave.)
1								
2								
3	0.15 to 0.3	1,010	1,115	1,181	1,213	1,206	1,254	1,235
4								
5								
6								
7	1.0	1,003	1,172	1,255	1,015	1,115	1,288	1,243
8								
9								
10								
11	1.5	1,050	1,142	1,166	1,184	1,160	1,161	1,170
12								
13								
14								
15	2.0	985	1,180	1,236	1,088	1,081	1,155	1,203
16								
17								
18								
19	2.5	995	1,150	1,180	1,145	1,177	1,200	1,271
20								

In a drylot feeding experiment with dairy cows for four years, Phillips, Hart, and Bohstedt [18] observed no effect on growth or maintenance of body weight when fluorine was fed as rock phosphate at a level of 2 to 3 mg. per kilogram of body weight. Elmslie [3] maintained 2 dairy cows under conditions of practical farming for five and one-half years on 1.2 to 2.1 mg. of fluorine, as rock phosphate, per kilogram of body weight, with no adverse effect on growth or weight. It remains to be seen if the continued feeding of fluorine as sodium fluoride for periods of more than three years at levels as high as 2.5 mg. of fluorine per kilogram of body weight will cause a decrease in the weight of dairy cattle.

General Health.—The over-all condition of the animals has remained satisfactory throughout the three years of the experiment. Although some unthriftiness has been observed during the peak of the milking periods of the cows which receive 2.5 mg. of fluorine per kilogram of body weight per day, it was a transitory condition. At no time was there any difference in general health between the cows fed the lower levels of fluorine, and the controls.

Two cows have been lost from the experiment, for reasons other than the ingestion of fluoride. Six months after the beginning of fluoride feeding, cow 15 was accidentally cast into an irrigation ditch and died. The third and fourth pairs of incisors of this cow had not erupted, but in their formative state the third pair appeared normal, while the fourth pair exhibited some hypoplasia. Cow 6 was killed in 1952 after several months of poor health. Postmortem examination revealed multiple abscesses of the spleen, due to the penetration of a foreign body. Other than tooth changes, no abnormalities were evident which could be ascribed to the continuous ingestion of the fluoride for almost two and one-half years.

The literature contains reports that the overgrowth of the toes of cattle is evidence of fluorosis. Throughout the periodic examinations of the herd, there has been no evidence of toe elongation in any of the animals. Neither has there been any diarrhea, rough coat, or lapping of water by the animals, as reported elsewhere.

Effect on Teeth.—Periodic examinations of the incisor teeth of the cows have proved useful in following the changes which occur when known amounts of fluorine are fed over long periods of time. At the beginning of the experiment, cow 10 had three pairs of permanent incisors. The first or second pairs of incisors in the other cows were formed, whereas the third and fourth pairs, and in some cows the second pairs, were in the formative or eruptive stage. It

TABLE 5—Condition of Incisor Teeth of Cows Fed Sodium Fluoride, After One, Two, and Three Years on Experiment

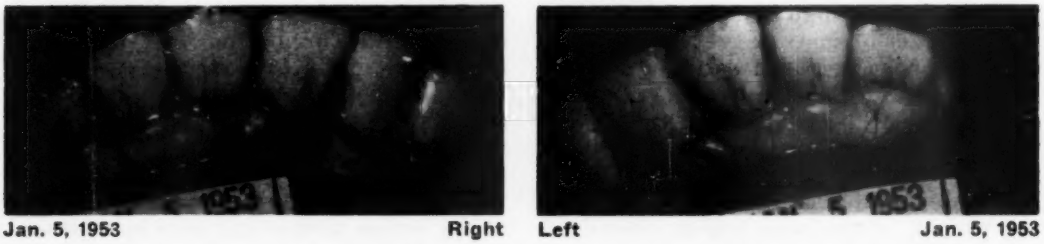
Cow (No.)	Total fluorine in diet (mg./kg. body wt./day)	First pair			Second pair			Third pair			Fourth pair		
		2/1/51	2/1/52	2/7/53	2/1/51	2/1/52	2/7/53	2/1/51	2/1/52	2/7/53	2/1/51	2/1/52	2/7/53
1	0.15 to 0.3	N	N	N	N	N	N	N	N	N	N	N	N
2	0.15 to 0.3	N	N	N	N	N	N	N	N	N	N	N	N
3	0.15 to 0.3	N	N	N	N	N	N	N	N	N	N	N	N
4	0.15 to 0.3	N	N	N	N	N	N	N	N	N	N	N	N
5	1.0	N	N	N(?)	N	N	N	vsD	vsM	vsM	T	S,M	vsM,sS
6	1.0	N	N	....	N	N	....	N	N	....	N	vsM,vsS	....
7	1.0	N	N	N	N	N	N	N	vsM	N(?)	E	vsM	vsM
8	1.0	N	N	N(?)	N	N	vsM	N	vsM	vsM	E,D	sM,vsS	sM,vsS
9	1.5	N	N	N	N	N	N	N	N	N	E,vsD	sM,sS	sM,vsS
10	1.5	N	N	N	N	N	N	N	N	N	N	vsM	vsM
11	1.5	N	N	N	N	N	N	N	vsM	N(?)	T	mM,mW,mS	mM,mW,mS
12	1.5	N	N	N	N	N	N	N	vsM	vsM	T	mM,sW,sS	mM,sM,mS
13	2.0	N	N	N	N	N	N	N	vsM	N	T	mM,sW,sS	mM,mW,mS
14	2.0	N	N	N	N	N	N	N	vsM	vsM	T	mM,sW	mM,mW,mS
16	2.0	N	N	N	N	N	N	N	vsM	vsM	T	mM,mW,mS	mM,mW,mS
17	2.5	N	N	N	N	N	N	N	vsM	vsM	E,D	mM,mW,sS	mM,mW,mS
18	2.5	N	N	N	N	N	N	sS	sM,vsS	sM	T	mM,mW,mS	mM,mW,mS
19	2.5	N	N	N	N	N	N	D	sM,vsS	sM,sS	T	mM,mW,mS	mM,mW,mS
20	2.5	N	N	N	N	N	N	N	N	N	D	mM,mS	sM,sS

T—temporary; E—erupting; M—mottling; W—wear; N—normal; D—dull; S—stain.
vs—very slight; s—slight, slightly; m—marked.

is generally accepted that dental hypoplasia occurs only during tooth formation. Thus the adverse effects of fluorides would be expected to occur in only those teeth that were not fully formed. Regular examination of the teeth at thirty-day intervals has shown that the maximum effect of fluorine on the incisor enamel occurs at a period approximately nine to twelve months prior to eruption. In table 5, the condition of the teeth after three years on experiment (Feb., 1953) is compared with the condition after two years (Feb., 1952) and after one year (Feb., 1951) on experiment. Photographs of representative cows from each group, taken at the start of the experiment and after one, two, and three years, may be seen in plates 1 and 2.

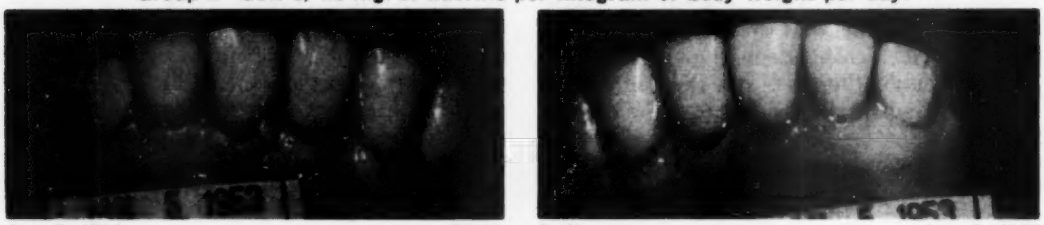
The term "normal," as used here, includes all conditions not associated with fluoride lesions found in the teeth of cattle. It means normal so far as fluoride effect is concerned. Actually, there are a great many abnormalities found "normally,"

Group 1—Cow 2, control.



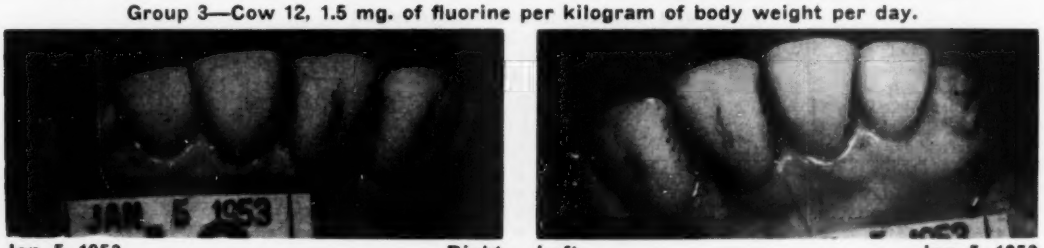
Jan. 5, 1953 Right Left Jan. 5, 1953

Group 2—Cow 8, 1.0 mg. of fluorine per kilogram of body weight per day.



Jan. 5, 1953 Right Left Jan. 5, 1953

Group 3—Cow 12, 1.5 mg. of fluorine per kilogram of body weight per day.



Jan. 5, 1953 Right Left Jan. 5, 1953

Group 4—Cow 14, 2.0 mg. of fluorine per kilogram of body weight per day.

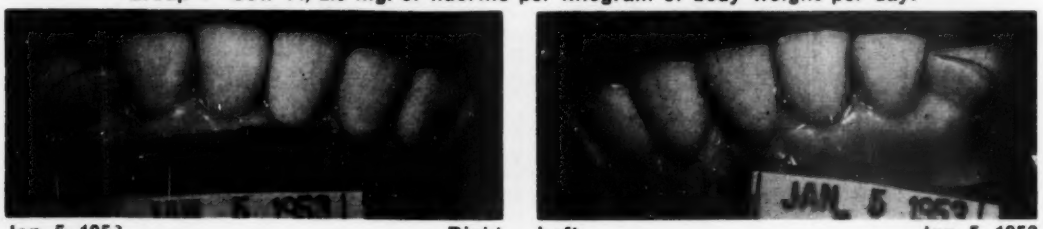


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Fig. 1—Incisor teeth of cows fed controlled amounts of sodium fluoride for three years.

and there is often great variation due to environment and breeding. However, within the scope of this project and with only 4 control animals, no attempt has been made to classify these various conditions. The terms used to describe changes in the teeth due to the ingestion of fluorine will be discussed in a paper to be published in the near future.

Two and one-half years after the start of the experiment, very slight mottling was observed in the second pair of incisors of cow 8, fed 1.0 mg. of fluorine as sodium fluoride per kilogram of body weight per day. There was no observable extension of this condition during the next six months. All other second incisor teeth have remained normal.

After one year, the third pairs of incisors of cows 5 and 19 were dull in appearance. By the end of the second year, very slight to slight mottling of the third incisors was observed in some cows of all experimental groups but it did not increase during the third year. Staining of these incisors has been variable, and is clearly evident in only 2 cows of the group which received 2.5 mg. of fluorine; cow 18 had a slight staining after one year, which has now disappeared, and cow 19 developed a very slight staining after two years, which has persisted and increased slightly since.

As was expected, the fourth pairs of incisors have shown the most marked effects of fluoride feeding. Although one half of the experimental cows still had temporary fourth incisors after one year of study, other cows in the groups fed 1.0, 1.5, and 2.5 mg. of fluorine had newly erupted teeth with a dull cast, suggestive of mottling beneath the encasing cuticle. By the end of the second year, a differentiation in the amount of mottling on the fourth pairs was apparent. Those cows receiving either 2.0 or 2.5 mg. of fluorine per kilogram of body weight per day showed marked mottling; those fed 1.5 mg. had slight to marked mottling; those which received 1 mg. had developed only very slight or slight mottling. Staining of the fourth incisors was

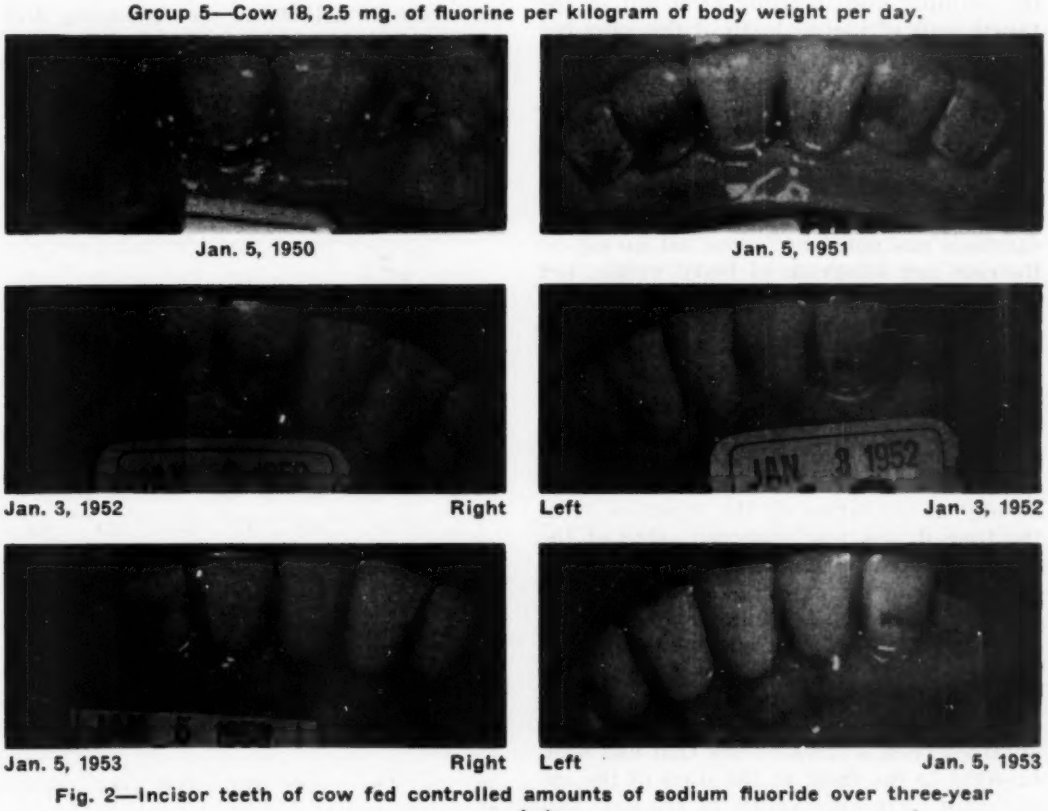

Fig. 2—Incisor teeth of cow fed controlled amounts of sodium fluoride over three-year period.

evident in all groups after the second year of the experiment, with the intensity of stain somewhat parallel to the amount of fluoride fed. Marked wear of the fourth incisors was evident after the second year in the group fed 2.5 mg. of fluorine, whereas those fed 1.5 or 2.0 mg. of fluorine showed slight to marked wear.

TABLE 6—Bone Fluorine Values of Cows Fed Sodium Fluoride

Cow (No.)	Total fluorine in diet (mg./kg. body wt./day)	Time on experiment (months)	Bone source	Fluorine p.p.m. (dry, fat-free)
15	2.0	6	metacarpal	1,040
6	1.0	28	metacarpal	1,050
			13th rib	2,020
Farm control	0.15-0.3	....	13th rib	588
6	1.0	22	13th rib	2,940
10	1.5	22	13th rib	3,430
14	2.0	22	13th rib	3,590
19	2.5	22	13th rib	5,000

p.p.m.—parts per million.

During the third year of the experiment, the staining and mottling observed in the fourth pair of incisor teeth of the cows fed 1.0 mg. of fluorine did not show any appreciable increase. In the groups receiving higher amounts of sodium fluoride, the fourth pair of incisors showed intensified staining, mottling, and increased amounts of wear.

Effect on Bones.—A mild condition of exostosis was noted in 2 cows fed 2.5 mg. of fluorine per kilogram of body weight per day, after the experiment had been under way for about a year and a half. X-rays were taken of the metatarsal bones of all the cows after twenty-eight months. The results revealed a slight thickening of the metatarsal in an additional cow fed the highest level of fluoride. After three years of the experiment, there does not appear to be any accentuation of the exostosis from the time it was first observed. One of the cows exhibited a slight limp during the second winter of the experiment, but the condition disappeared the following spring.

Biopsy samples were obtained from the thirteenth rib of 1 cow in each of the experimental groups after twenty-two months of fluoride feeding. A control sample was obtained from a Lavacre cow that had been brought to the farm at the start of the experiment. Fluorine values of the dry, fat-free bone samples reflected the relative ingestion levels of fluoride. These results, in addition to metacarpal values of 2 cows lost during the experiment, are given in table 6.

Urinary Excretion of Fluorides. — The urinary excretion of fluorine has consistently reflected the relative intake of sodium fluoride by the experimental herd. Urine samples taken eight to twelve hours after the daily dose of sodium fluoride was fed have shown a variable increase in fluorine over samples taken twenty-two to twenty-four hours after ingestion of the supplements. The fluorine values of the urine as presented in table 7 were obtained from single samples collected just prior to the daily feeding of sodium fluoride. Despite the obvious variation of individual cows within each group, a relative pattern of excretion has been established and continued throughout the experiment.

SUMMARY

We emphasize that this is a progress report of a study in which an attempt is being made to determine the damaging and nondamaging boundary levels of fluorine when fed under practical farm conditions to dairy cattle in the soluble form as so-

TABLE 7—Fluorine Concentration in the Urine in Parts per Million from Cows Fed Sodium Fluoride (Fluorine Values Adjusted to Specific Gravity of 1.04)

Cow (No.)	Total fluorine in diet (mg./kg. body wt./day)	12/28/49 Group (ave.)	12/12/50 Group (ave.)	12/17/51 Group (ave.)	8/3/52 Group (ave.)	12/12/52 Group (ave.)
1						
2						
3	0.15 to 0.3	0.9	1.7	2.3	1.4	2.5
4						
5						
6						
7	1.0	0.8	7.6	13.0	7.1	9.3
8						
9						
10						
11	1.5	0.9	10.7	14.7	11.7	11.0
12						
13						
14						
15	2.0	0.5	16.6	11.9	12.3	12.9
16						
17						
18						
19	2.5	0.9	21.1	22.8	18.7	25.4
20						

dium fluoride. The results obtained after three years of experimentation may be summarized as follows:

1) The daily ingestion of fluorine as fluoride at levels as high as 2.5 mg. per kilogram of body weight, by dairy cows through two lactations, did not reduce milk production significantly and failed to cause a depression in weight.

2) There has been no evidence in any of the cows of diarrhea, rough coat, lapping of water, or elongation of the toes which might be attributed to the prolonged ingestion of fluorides.

3) The fourth pairs of incisor teeth in cows fed 2.0 and 2.5 mg. of fluorine per kilogram of body weight have shown marked mottling, marked staining, and marked wear. At the 1.5-mg. fluorine level, the degree of mottling, staining, and wear of the fourth incisors was less. Only slight mottling and staining of the fourth pairs of incisors, with no wear, was evident in the group fed 1 mg. of fluorine per kilogram of body weight. These pathological conditions were less frequent and less intensive in the third incisors. The first and second incisors of all cows, except 1, have remained free of any tooth changes.

4) Slight exostosis has developed in 3 of the 4 cows fed 2.5 mg. of fluorine per kilogram of body weight but not in cows fed lower levels of fluorine.

5) The measurement of urinary excretion of fluorine has proved to reflect the relative levels of ingestion of fluorine.

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Observations on Immunity in Cutaneous Bovine Papillomatosis

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ALTHOUGH wart vaccines have been commercially available for years, the literature contains no information on controlled tests of the immunity they induce against papillomatosis in cattle.

Papillomatosis occurs naturally most often in cattle under 2 years of age. The disease seldom occurs in older animals which appear to be more resistant. This resistance is generally attributed to infection with papillomas in calfhood which renders the animals immune.

Transmission experiments with the different agents causing cutaneous papillomatosis (warts, verrucae) in different species of animals have demonstrated variations in susceptibility. The exposure of cattle under 2 years of age,[1-5] horses under 3 years of age,[6] and young dogs[7] resulted in formation of growths. On the other hand, older cattle and horses[5,6] appeared to be somewhat resistant to the infection since experimental exposure of older animals was not always successful. Cattle,[2] horses,[6] dogs,[7] and rabbits,[8] recovered from natural or experimental disease, remain partially or completely immune to a subsequent exposure.

The basis for this report is a study of the immunity following the use of two commercial wart vaccines in a herd of young cattle. The duration of immunity following the natural and experimental infection was also studied in two groups of cattle.

METHODS

Two commercial wart vaccines were used for immunization. One consisted of ground bovine papilloma tissue and the other was material from chicken embryos which had been inoculated with bovine papilloma material. Both had presumably been inactivated with formaldehyde. Each of the animals selected for immunization received two 20-ml. doses of vaccine subcutaneously two weeks apart. The challenge of immunity was made eighteen to nineteen days following the last injection of wart vaccine by inoculation with suspensions of glycerinated tissues containing active wart virus.

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Ground tissue suspensions were made by using from 7 to 15 cc. of physiological saline solution to 1 Gm. of wart tissue. Inoculum A was prepared from a case of papillomatosis which occurred on an Iowa farm in April, 1949. Inoculum B was made from warts removed from a steer at Lincoln, Neb., in June, 1951. Inoculum C contained tissue of warts from 5 cows kept in the herd at Valentine, Neb., where this experiment was conducted. Inoculums D, E, F, G, H, J, and K were prepared from warts collected from different cattle in three herds near Lincoln during the period from January, 1951, to September, 1952. Inoculum L was made from an experimental wart produced by inoculum A in a calf in the herd at Valentine. All inoculations were made intradermally and upon scarified skin in duplicate with approximately 0.2 ml. of inoculum. The animals were examined at intervals to measure the size of the growths and note their characteristics. Several biopsies were made for histological examination.

Experiment 1.—A herd of 82 calves was divided into three groups according to age so that each group contained animals of comparable ages varying from 1 week to 9½ months. One group of 27 calves received a vaccine of tissue origin. A second group of 28 animals was given vaccine of chicken embryo origin. The third group of 27 animals remained untreated and served as controls. The animals selected for immunization received the respective vaccines on May 24, 1951, and again on June 6, 1951. All 82 calves received the challenge exposure of inoculums A, B, and C, containing active wart-virus material eighteen to nineteen days following last injection of vaccine.

RESULTS

No naturally occurring warts developed on these animals during the 115 days of observation under this experiment. Reactions to the different strains of wart-virus

TABLE 1—Percentage of Calves Which Reacted to Inoculation with Three Strains of Wart Material as a Challenge of Previous Immunization with Commercial Vaccine

	Group		
	1	2	3
Strain	27 received tissue vaccine	28 received chicken embryo vaccine	27 controls
A	63*	96	100
B	56	96	89
C	30	93	82

* Expressed as percentage of group in which a reaction occurred.

material occurred in all groups of calves (table 1). The calves receiving tissue vaccine prior to exposure showed the least response. The challenge to calves which had received chicken embryo vaccine indicated no protective effect when compared to the group of control calves.

TABLE 2—Type of Growth Resulting from Challenge Inoculation

Immunized with	Mode of inoc.*	No. growths	Type of growth 1 papilloma	2 nodular	3 swelling
Tissue vaccine	id.	67	0	72**	28
(27 calves)	scar.	4	0	25	75
Chicken embryo vaccine	id.	154	4	88	8
(28 calves)	scar.	53	75	12	13
Controls	id.	133	2	87	11
(27 calves)	scar.	39	69	26	5

* id. = intradermal; scar. = scarified; ** = percentage of number of growths observed.

Three types of growth response followed the challenge inoculations. Type 1 resembled the natural papilloma and projected above the skin in the shape of a cauliflower-like growth with fine fissures on the surface (fig. 1, a, b, c). These growths occurred most frequently at the scarified inoculation sites (table 2). Histologically, these growths consisted principally of hyperplastic epithelium resting on proliferating connective tissue (fig. 2). The dermal papillae were sometimes irregular in length. The prickle cell layer was thickened (acanthosis) (*see* fig. 3). Mitotic figures were fairly common in the basal layer and in the lower portion of the prickle cell layer. In the outer portion of the prickle cell layer, there were a few swollen and degenerated cells with clear areas in the cytoplasm. The connective tissue response under the hyperplastic epithelium was variable.

The type 2 growth was a well-circumscribed, firm nodule localized in the skin at the site of inoculation. The nodules varied in size but were definitely elevated (fig. 1, A, B, C). These could be readily measured. They occurred most frequently at the sites of intradermal inoculation (table 2). Histologically, the epithelium covering the nodules displayed only a moderate disturbance of the prickle cell layer with some thickening of the horny layer (fig. 4). At the edges of the lesion, the dermal papillae were short and thin as they projected into the connective tissue. In the central portion of the lesion, the epithelium was flattened and thin. Sometimes there were small groups of epithelial cells forming islands in the subepithelial connective tissue and may have represented remnants of dermal papillae. Occasional mitotic figures were observed in these cells. The principal mass of the growth was connective tissue which had replaced or pushed aside the normal structures of the corium. The nodules of connective tissue were well circumscribed.

The type 3 growth was characterized by a swelling. It was palpable as a small, firm nodule in the skin which caused only a slight elevation. These growths were difficult to measure and were most common in the group of calves previously immunized with tissue vaccine (table 2). In these small reactions were localized areas in which the connective tissue of the stratum reticulare was infiltrated with bundles of

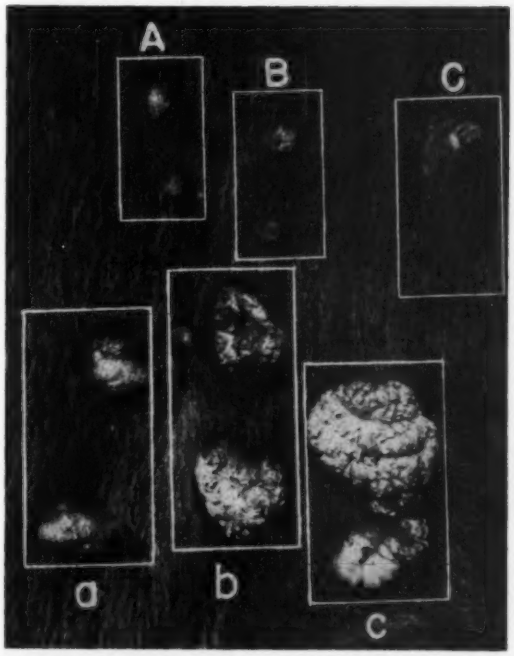

Fig. 1 — Experimental growths fifty-nine days after inoculation with three strains (A, B, C) of bovine papilloma agent. Areas A, B, and C illustrate the nodular type of growth produced by intradermal inoculation. Areas a, b, and c represent papilloma-like growths which followed inoculation of scarified skin.

more densely staining connective tissue fibers. These bundles were rich with nuclei. In some instances, the stratum papillare was heavily infiltrated with polyblasts.

TABLE 3—The Response of 58 Cows to Inoculations with Three Different Strains of Wart Material

Strain	Positive (%)
A	53
B	69
C	14

Correlation between the type of growth reaction and previous immunization indicated that only the tissue vaccine had an effect (table 2). Relatively fewer growths occurred in this group of animals and none of them were of the papillomatous type.

Most of the growth reactions were present thirty-five days after inoculation. Very few had an incubation period of more than fifty-nine days. There was no marked difference in the incubation period of the reactions induced by intradermal inoculation compared to that following scarification.

Most of the lesions disappeared in less than ninety days (27 % existed less than 24 days, 27 % lasted from 30 to 60 days, 27 % lasted from 60 to 90 days, and 19 % lasted from 90 to 180 days). There was no correlation between duration of the lesion and method of inoculation or previous immunization.

Fig. 2—Section from papilloma-like growth (fig. 1, c) to show the hyperplastic epithelium. This covers dermal papillae whose normal structure has been replaced by new connective tissue. x 17.

Experiment 2.—The duration of immunity following the natural disease was tested in 58 cows 3½ years old. Papillomatosis in enzoötic form had been present in the herd since May 12, 1949.[9] There were two episodes of papillomatosis, one in 1949 and the other in 1951. Forty-six animals, 79 per cent of the herd, were affected with the disease once. Natural reinfection occurred in 16 of these animals in 1951 (prior to experimental exposure).

The immunity of these animals was challenged by intradermal and scarified skin exposure with inoculums A, B, and C in June, 1951.

RESULTS

The growth response was only a slight swelling which possessed the same characteristics as type 3 described in experiment 1. This reaction occurred at the sites of intradermal inoculation in 76 per cent of the animals. Inoculation upon scarified skin produced only two growths. The least response was to inoculum C which originated in this herd (table 3). None of the cows developed the epithelial reaction of typical papillomas (type 1).

The response to the inoculations could be correlated with previous natural infection. The group of 16 animals with two previous natural infections of papillomas in 1949 and 1951 appeared more resistant than those with a single previous infection (table 4). Natural papillomas were observed in 3 animals which did not respond to the experimental inoculations.

Most growths were evident thirty-five days following the inoculation. About 12 per cent more were first observed on the fifty-eighth day. Most growth reactions lasted less than twenty-four days. Thirty-seven per cent lasted from thirty to sixty days and in only a few cases did they persist for ninety days.

Experiment 3.—The duration of immunity following the experimental inoculation of animals with active wart material subsequent to treatment with wart vaccines was tested in 18 calves carried over from experiment 1. When tested, they were from 17 to 24 months old. Four of them had received vaccine of tissue origin, 8 received wart vaccine of chicken embryo origin, and 6 were untreated controls. Intradermal exposure of these animals with inoculums B, D, E, F, G, H, J, K, and L was made Sept. 30, 1952 (462 days after

previous challenge). At this time, 6 animals were affected with natural papillomas.

Result

A mild reaction of the connective tissue (type 3) was found in 13 of the 18 animals. No typical papillomas developed. There was no uniformity of reaction to the different inoculums. Four animals reacted to eight of the nine inoculums and others to only one, two, four, or six inoculums. The variation of reactions could not be correlated with the previous history. Four of the 6 animals with natural papillomas at the time of experimental exposure did not react.

TABLE 4—The Susceptibility of Animals to Experimental Inoculation Correlated with Year of Previous Natural Infection

Infection with papillomatosis	No. animals	Susceptible (%)
Negative	12	75
1949	15	93
1951	15	86
1949 and 1951	16	50

Discussion

The attempt at active immunization of susceptible animals with two commercial wart vaccines demonstrated a difference in the immunizing efficiency of the two vaccines when the animals were exposed to subsequent experimental infection. Vaccine of tissue origin appeared to have more ability to immunize, in this experiment, than the vaccine of chicken embryo origin. Complete immunity, however, was not obtained. Differences in the response to the three strains of wart material used for challenge in experiments 1 and 2 suggest some specificity of the immunity. Apparently the vaccine of tissue origin was prepared from warts containing virus immunologically related to those present in inoculums. However, little is known about the existence of different strains of the agent of cutaneous papillomatosis and their activity or immunological properties. Two factors may have been responsible for the lack of an immunizing property in the chicken embryo vaccine. An immunologically unrelated strain may have been used for its production or there could have been loss of its immunizing ability while growing in the chicken embryo.

Recovery from natural infection did not produce complete immunity against subsequent natural or experimental infection. Some animals which had the disease in 1949 became reinfected in 1951.[9] Similar reinfection with warts in man was described by Rulison.[10] Seventy-six per cent of 58 cattle from the herd in which papillomatosis had been present for two years were still susceptible to experimental infection by developing reactions of the connective tissue. The degree of susceptibility in these animals, if measured according to the type of growth, was considerably less than in the young susceptible calves of experiment 1. Relatively more susceptible

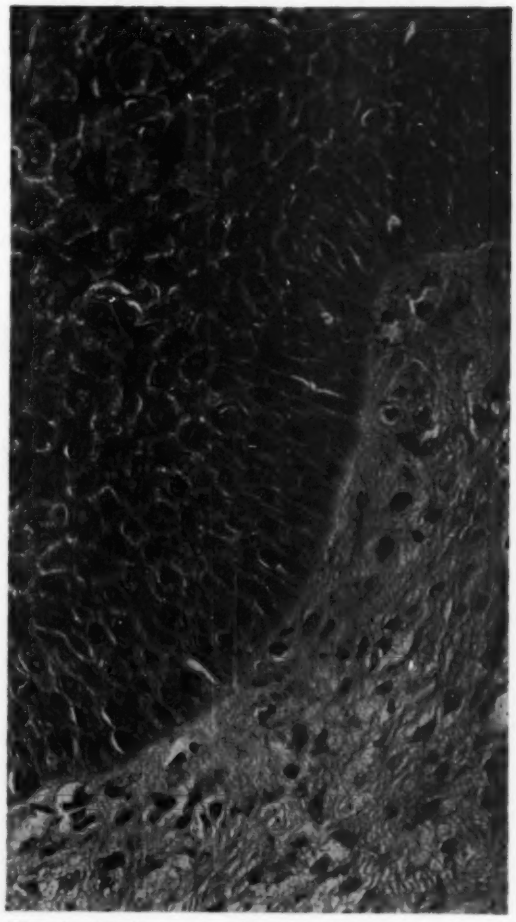

Fig. 3—Higher magnification of area 1, figure 2. Thickening of the prickle cell layer may be noted. The sharp border between the hyperplastic epithelium and proliferating connective tissue is also evident. x 300.

animals were observed among those which had a single episode of disease. It could be assumed, therefore, that those with two previous infections were more immune to experimental inoculation. Even this immunity, however, did not protect against natural infection. The development of natural warts in a horse recovered from experimental infection was observed by Cook and Olson.[6]

Olson and Cook [11] found that the agent of bovine papillomatosis could markedly stimulate connective tissue in the corium of the horse. McEntee [12] has reported that the agent of bovine papillomatosis could induce a connective tissue response in cattle causing fibropapillomas of the external genitalia. The connective tissue reaction was also illustrated by Creech [1] in experimental bovine warts. Histological examination in the course of the present work demonstrated the ability of the agent to induce proliferative changes in both epithelial and connective tissue of cattle.

Growths produced by intradermal inoculations or upon scarified skin of the susceptible young animals developed into comparatively large tumors resembling cutaneous papillomas both clinically and histologically. Only growths of connective tissue developed in young animals previously immunized with vaccine of tissue origin (table 2), thus indicating immunity of epithelial tissue. This was also true for older animals. The connective tissue growths in experimentally immunized animals were smaller in size when compared with those of susceptible animals. In older animals recovered from the infection, they were often so small that they could easily have been missed. Inoculation upon the scarified skin of experimentally immunized young animals, and older animals recovered from natural or experimental infection, failed to induce any detectable growth.

Fig. 4—Section from a nodular type of growth (fig. 1, C). The connective tissue proliferation is evident. Some hyperplasia of epithelium has developed near the periphery of the nodule. x 17.

The experimental immunization of susceptible young animals, and recovery from natural or experimental infection in older animals, evidently rendered the epithelium immune to infection. The rare occurrence of cutaneous papillomas in older animals supports these observations. This immunity, however, is not absolute and does not always protect against natural infection since natural papillomas did occur in animals which were immune to the experimental exposure.

SUMMARY

The immunity following the injection of commercial wart vaccines of tissue and chicken embryo origin was studied in 82 young calves. The immunity was challenged by intradermal and scarified skin inoculation with three preparations of ground wart tissue containing active virus. Three types of growth were produced. One was typical of the epithelial papilloma with some connective tissue reaction. The second and third types consisted principally of a connective tissue response which differed only in degree. A partial degree of immunity was produced in calves immunized with wart vaccine of tissue origin. No protection was demonstrable in calves which received wart vaccine of chicken embryo origin. The duration of immunity was tested in 18 animals more than a year after experimental immunization and challenge. Only a connective tissue response was obtained in 13.

Some animals in a herd of 58 cows (3½ years old), recovered from or still affected with natural papillomas, developed connective tissue response after experimental

exposure. The animals that had recovered from two attacks of the disease showed less response of connective tissue than those recovered from a single episode of the infection.

Some evidence suggests strain specificity with respect to immunity.

While both epithelium and connective tissue were stimulated by the agent of bovine papillomatosis, only the epithelium appeared to become distinctly immunized as a result of experimental immunization or natural infection.

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An Inflatable Plug for Small Rumen Fistulas

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MANY kinds of plugs for closing the openings of rumen fistulas have been devised and are successful. Many of them have been constructed for openings in the cow large enough to admit the arm. There are times, however, when a small fistula is all that is necessary to obtain the desired data. There are times when a plug for these fistulas is required, which can be removed easily and which can be inflated momentarily to seal the fistula opening. The accompanying photograph illustrates this type of plug. It has worked well in small rumen fistulas in sheep and has withstood rumen pressures of several pounds per square inch without leakage. Most machine shops can construct the base of the plug from the accompanying illustration. Its size can be variable. Most plastics are satisfactory. The inflatable portion is part of an ordinary toy balloon. The air passage to the inside of the balloon is a small hole extending at an angle from the rubber tubing adapter hole to the deep groove of the stem inside the balloon.

Fig. 1—Inflatable plug for small rumen fistulas.

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Bactericidal Activity of the Uterus in the Rabbit and the Cow

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Madison, Wisconsin

PREVIOUS work at this laboratory [1] has demonstrated a differential response in rabbits of various endocrine states following intra-uterine instillation of bacteria. Similarly, an apparent difference between luteal-phase "first-service" heifers and "repeat-breeding" cows has been noted in the degree to which pyometra is induced by such procedures.[2] The present work was undertaken to determine whether these differences in response, both in terms of the gross appearance of pus and of histological changes, are reflections of differences in the bactericidal or bacteriostatic activity of such uteri. The technique used in this determination has been one of culturing quantitatively a bacterium in the uterus of the living animal.

EFFECT OF ENDOCRINE STATE ON BACTERIAL SURVIVAL IN THE RABBIT

Previous experiments [1] have shown that uteri of rabbits in estrus and of spayed rabbits evidence only small amounts of pus or cellular débris following inoculation with *Escherichia coli*, while histological examination shows increased numbers of polymorphonuclear leukocytes in the endometrium. Pseudopregnant rabbit uteri, on the other hand, respond with a heavy accumulation of pus in the lumen and a massive leukocytic infiltration of the uterine tissues. That this difference is not entirely owing to differences in cervical drainage resulting from differences in uterine motility was shown by the failure of typical pyometra to be produced in rabbits in estrus in which the cervices were ligated prior to inoculation to prevent drainage.

In the present test, emphasis was given to the fate of the bacteria constituting the inoculum, rather than to the changes produced in the uterus.

METHODS

Females, spayed, pseudopregnant, and in estrus, were used in replicated trios. Spayed rabbits were obtained by performing a bilateral ovariectomy on females in estrus fifteen days prior to treatment; pseudopregnant does were secured by mating females in estrus to vasectomized males ten days prior to treatment. On the day of treatment, 1 rabbit in each of the three endocrine states was anesthetized and a midventral laparotomy performed. An incision was made through the anterior vaginal wall and the protruding portion of the right cervix ligated with nylon ligatures to prevent drainage. Following this, 0.1 ml. of a fresh saline suspension of *E. coli* organisms (washed from slopes of actively growing cultures) was injected into the lumen of each uterine horn by puncturing the uterine wall at the anterior end. Thus, each horn of the 3 rabbits constituting one replicated trio received approximately equal numbers of organisms. The number of bacteria comprising the inoculum for each of 6 replicates was determined by plate count and found to vary between 3 and 15 million cells.

The rabbits were destroyed twenty-four hours after treatment and the reproductive organs removed. The cervix of each uterine horn was discarded and the uterine contents recovered in test tubes by alternately flushing with physiological saline and stripping. Ten milliliters of washings were collected from each horn as aseptically as possible. This fluid, together with any accumulated pus, was mixed and aliquots plated serially on nutrient and eosin-methylene blue agar. After forty-eight hours' incubation, colonies showing characteristics of *E. coli* were counted to obtain estimates of the number of organisms still resident in the uterus.

Contamination by extraneous microörganisms during recovery of the uterine lavage did not seriously interfere with the plate counts; a more important failing was the selection, in the case of several rabbits, spayed and in estrus, of serial dilutions too high to provide accurate estimates of

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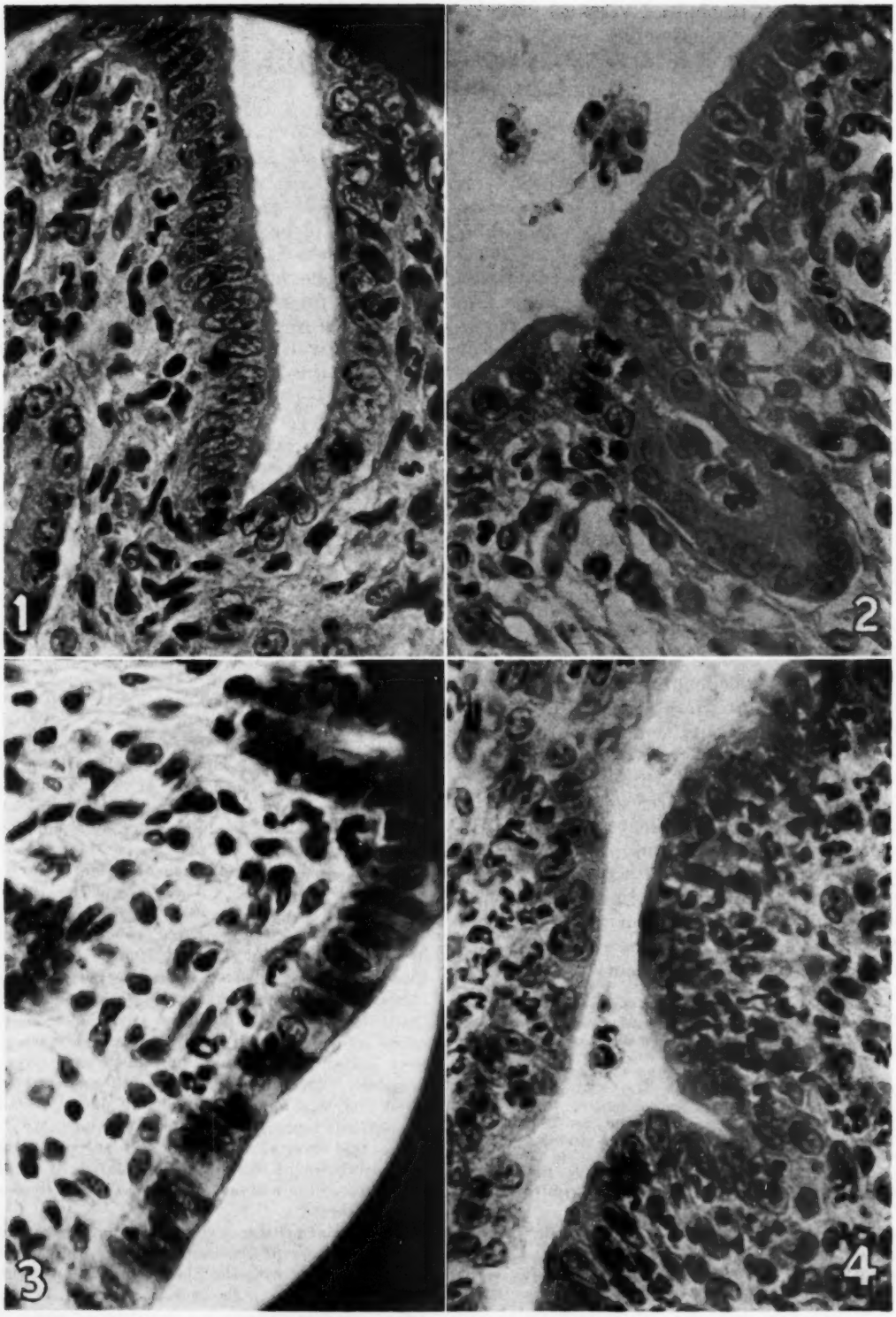
The authors express their appreciation for the assistance of T. Kowalczyk, H. Cohen, and L. Wipf in the surgical, bacteriological, and histological aspects of the study, and of S. H. McNutt and L. E. Casida in the planning and conduct of the work.

Agents of the Bureau of Dairy Industry (Black, Kidder, Wiltbank); Department of Veterinary Science (Simon).



Uterine sections of representative rabbits destroyed twenty-four hours following intrauterine instillation of Escherichia coli. x 650.

the numbers of bacteria involved. In consequence, the tabular values given tend to overestimate the actual numbers.

RESULTS

A highly significant difference was found (table 1) between animals of the three endocrine states in the numbers of bacteria recovered (analysis of variance of logarithms of counts). This difference was entirely due to the low degree of bactericidal activity in the pseudopregnant animals. In several of the latter rabbits, more bacteria were recovered than introduced. Also, differences between ligated and unligated horns did not prove significant in such rabbits.

TABLE 1—Mean Number of Bacteria Recovered from Inoculated Uteri of Rabbits of Various Endocrine States (6 Replicates, Inoculum Varying Between 3 and 15 Million Bacteria).

Type of rabbit	Uterine Horn	
	ligated	unligated
Estrous	<105,122	< 27,353
Spayed	<180,028	<166,785
Pseudopregnant	7,011,167	7,225,000

Ligation of the cervix in spayed animals and animals in estrus in general led to greater survival of the bacteria, presumably by preventing natural drainage of the uterine contents. This view is supported by the fact that a small amount of pus was found in almost all ligated horns in these animals but not in the unligated horns. Ligation in animals in the luteal phase, however, appeared to produce no additional retention of organisms or pus.

Histological examination of uterine sections of these rabbits showed the degree of inflammation present to be roughly associated with the estimates of surviving bacteria. Microphotographs of representative specimens in this series are presented in figures 1 through 6.

BACTERICIDAL ACTIVITY OF THE BOVINE UTERUS

Earlier work [2] demonstrated that a parallel difference in susceptibility to pyometritis exists in the cow at corresponding stages of the estrous cycle. More recently, Rowson *et al.*[4] have confirmed these findings in cows inseminated with semen inoculated with *Corynebacterium pyogenes*. In the former study,[2] an apparent difference was also noted in the pus-forming activity of the uterus of first-service heifers and repeat-breeding cows following insemination during the luteal phase, the latter animals rather consistently exhibiting a lower grade reaction to the bacterial contaminants of the semen.

The technique of *in vivo* bacterial culture was again employed to determine whether there is a difference in bactericidal activity of these two kinds of cows.

METHODS

Eight first-service animals (7 virgin heifers and 1 uniparous cow) were obtained for this study, together with a comparable number of parous repeat-breeder cows, selected for their apparent infertility but otherwise clinically normal, as described by Tanabe and Casida [5] and Christian *et al.*[3]

One first-service and 1 repeat-breeding animal, at comparable stages of the estrous cycle (seven to eleven days postestrus), were subjected to simultaneous midventral laparotomy. The uteri were exteriorized and each horn ligated at the level of the external bifurcation to prevent drainage. Two milliliters of the same fresh saline suspension of *E. coli* were injected through the uterine wall into the lumen of one uterine horn above the ligature in each animal. The opposite horn, serving as a control, received 2 ml. of a saline solution washed from sterile agar slopes. Thus each member of a pair received approximately the same numbers of bacteria, the inoculum varying between 300 and 13,600 million bacteria.

The animals were slaughtered thirty-six to forty-eight hours after inoculation and the reproductive organs recovered. The ligated portion of each horn was flushed with 10 ml. of saline solution, the recovered washings shaken into uniform suspension, and aliquots plated on nutrient and eosin-methylene blue agar. After forty-eight hours' incubation, coliform colonies were counted

Legends for Illustrations on Opposite Page

Fig. 1—Endometrium of a rabbit in estrus, cervix not ligated. A few polymorphonuclear leukocytes are present in the mucosa.

Fig. 2—Endometrium of a rabbit in estrus, cervix ligated. Essentially the same as figure 1 except that a few leukocytes are present in the uterine lumen.

Fig. 3—Endometrium of a spayed rabbit, cervix not ligated. Only a few polymorphonuclear leukocytes are present in the mucosa. Reaction the same as in the rabbits in estrus.

Fig. 4—Endometrium of a spayed rabbit, cervix ligated. Considerable numbers of polymorphonuclear leukocytes in the mucosa and a few in the uterine lumen. Reaction more extensive but essentially the same as in the rabbit in estrus (fig. 2).

to obtain estimates of the number of bacteria surviving the *in vivo* culture. In addition, sections of each uterine horn, both anterior and posterior to the ligatures, were secured for histological study.

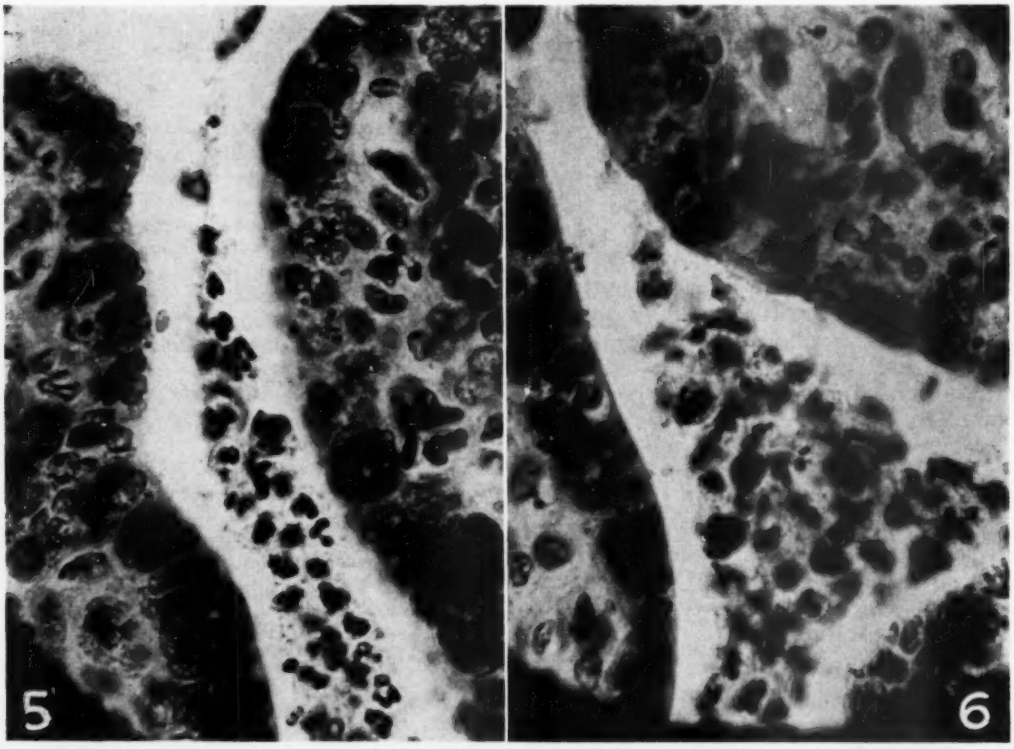
RESULTS

Eight pairs of animals were scheduled for this test. At the time of laparotomy, however, 2 of the repeat breeders exhibited gross evidence of endometritis and were therefore eliminated. Data included in table 2, in consequence, represent observations on 6 pairs of animals plus 2 unmatched first-service heifers.

Some difficulty was experienced in securing ligatures tight enough to prevent passage of the instilled bacteria into the control horn, particularly in the first-service group. Evidence of such leakage was also found on histological examination of these uteri by diagnosis of acute endometritis in regions other than the inoculated portion of the uterine horn. Nevertheless, the difference in the number of bacteria recovered from the inoculated horns in the two types of animals, while not significant statistically, is suggestive of a real difference in bactericidal activity. The exceptional case, in which more bacteria were recovered from the repeat-breeder than from the first-service pair mate, might well be expected with such an approximate technique and with animals rather arbitrarily classified as normal and infertile.

DISCUSSION

The uterus of the rabbit in estrus must possess a high degree of bactericidal activity as measured both by lack of extreme pathological changes following inoculation



Uterine sections of representative rabbits destroyed twenty-four hours following intrauterine instillation of Escherichia coli. x 650.

Fig. 5—Endometrium of a pseudopregnant rabbit, cervix not ligated. (It will be observed that the uterine endothelium of the pseudopregnant rabbit is vastly different from that of the spayed rabbits or those in estrus.) There is marked pyometra; polymorphonuclear leukocytes are abundant in uterine exudate.

Fig. 6—Endometrium of pseudopregnant rabbit, cervix ligated. The reaction is the same as in figure 5, with perhaps a little more exudate in the uterine lumen.

and the poor bacterial recovery. That this activity is not necessarily a function of estrogen is shown by the similar pattern in the ovariectomized female. As earlier work has shown,[1] the progesterone produced in the luteal phase of the estrous cycle is responsible for a complete or partial loss of this activity. It seems reasonable to the present authors, as well as to Rowson *et al.*,[4] that this loss of resistance may be allied with a changing uterine environment necessary for successful nutrition and implantation of the embryo.

TABLE 2 — Number of Bacteria Recovered from Uteri of Cattle of Normal and Impaired Fertility (Inoculum Varying Between 0.3 and 13.6 Billion Bacteria).

	First Service		Repeat Breeder	
Pair	Inoculated horn	Control horn	Inoculated horn	Control horn
1	400,000	10,000	80,000	0
2	690,000	2,000	1,500	< 500
3*	200,000	160	.....	....
4	400,000	< 100	< 100,000	0
5*	900,000	< 100	.....	....
6	1,900,000	1,780,000**	370,000	0
7	16,200,000	0	25,440,000	0
8	160,000	80	10,400	0

* Pair mate discarded because of preëxisting endometritis.

** Ligatures observed to be very loose at slaughter.

In the cow, a similar pattern is observed although, from a comparison of bacterial recovery in both the luteal-phase rabbit and cow, the defense mechanism in the latter animal is not suppressed to the same extent. The hypothesis that the endometrium of the repeat breeder generally possesses a greater bactericidal activity (or lower susceptibility to infection) than that of the first-service animal, as presented by Black *et al.*,[2] is given support by these findings. Whether this difference in response is the direct cause of the difference in fertility or only an associated phenomenon can not be determined with present techniques.

Attempts have been made, through the use of endometrial extracts,[6] to determine whether chemical agents in the uterus are directly involved in the destruction of bacteria. While the negative nature of these early trials does not preclude the existence of such substances too low in concentration to be measured, it is perhaps more likely that the action of progresterone in suppressing the natural defenses is mediated through the leukocytes, rendering them incapable of destroying the invading bacteria. In this connection, the role of opsonins in infection might bear further investigation.

SUMMARY

Comparisons were made of the numbers of bacteria recoverable from the uteri of estrous, spayed, and pseudopregnant rabbits inoculated twenty-four hours previously with known concentrations of *Escherichia coli*. Estrous and spayed females exhibited a high degree of bactericidal activity; ligation of the cervices in these animals tended to increase the number of surviving bacteria by preventing natural drainage. Bactericidal or bacteriostatic activity was practically nonexistent in pseudopregnant females.

The same technique was employed in comparing the uterine bactericidal activity of luteal-phase cows of normal and impaired fertility. "Repeat-breeding" cows generally exhibited a greater defense toward bacterial infection than did "first-service" animals, suggesting a possible cause of their lowered fertility.

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Electrophoretic Studies of Foot-and-Mouth Disease Virus

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Lindholm, pr. Kalvehave, Denmark, and Washington, D. C.

ALTHOUGH crude preparations of foot-and-mouth disease virus have been studied electrophoretically, purified preparations have not been studied extensively. In 1949, Janssen [1] described some experiments with crude guinea pig virus and also a few experiments with purified preparations. More recently Bachrach,[2] using a filter paper technique of zone electrophoresis, reported some observations on crude guinea pig adapted virus. The conclusions of these two studies are in agreement, but all observations were made near pH 7.6. The object of the present study was to extend these observations by determining the electrophoretic behavior of the virus under different conditions.

METHODS

All virus used in this study was of the O type produced *in vitro* by tissue culture techniques, such as those described by Frenkel.[3, 4] After the virus was harvested, it was concentrated by ultrafiltration on nitrocellulose membranes which retained all serum proteins as well as the virus.[5] Two to 5 liters were concentrated to about 200 ml. before the virus was sedimented by centrifugation. Practically all of the virus was found in the pellet after three and one-half hours at about 50,000 g (24,630 r.p.m. in the preparative D rotor of the model E Spinco ultracentrifuge*). The pellet was resuspended in 9 ml. of phosphate buffer pH 7.5 and sedimented again in the C rotor at comparable speeds. In some instances, the pellet was resuspended and sedimented a third time. The final pellet was resuspended in about 10 ml. of the phosphate or veronal buffer used for electrophoresis. The suspensions were clarified by slow speed centrifugation prior to filling the electrophoresis cell. Because the purified virus was rapidly inactivated on standing, even at 0 C., the preparations were not dialyzed. Not all of the final purified virus preparations were examined, but analytical ultracentrifugation showed representative preparations to be homogeneous.

For comparative purposes, virus-free cultures (referred to here as blind cultures) were treated in the same manner as were the virus cultures, except that they were not inoculated with virus prior to incubation. Such preparations should, therefore, contain the nonvirus substances which are present in the virus preparations cultivated *in vitro*.

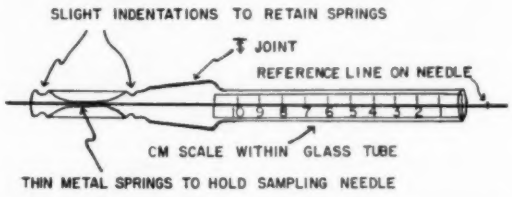
SLIGHT INDENTATIONS TO RETAIN SPRINGS
JOINT
REFERENCE LINE ON NEEDLE
10 9 8 7 6 5 4 3 2 1
CM SCALE WITHIN GLASS TUBE
THIN METAL SPRINGS TO HOLD SAMPLING NEEDLE

Fig. 1—A diagram of a device to hold a small steel tube for removing samples from an electrophoresis cell.

An Aminco electrophoresis apparatus** with the standard Tiselius cell (81 by 25 by 3 mm.) was used for all observations reported here.

The mobility of the infectious particles was followed by removing small samples from various levels in the cell at the end of the electrophoretic run. Accurate sampling was facilitated by the use of a device, described by Randrup,[6] consisting of a stainless steel tube with an outside diameter of 1.6 mm. and an inside diameter of 0.9 mm., with a close fitting wire which acted as a piston. The tube, with the wire in place, was inserted into the cell and the sample drawn into the tube by withdrawing the wire a measured distance. A small amount of heavy stopcock grease applied to the lower end of the tube prevented any fluid from entering the tube until the wire was withdrawn. Since the volume of the samples could be controlled to within 0.005 ml., samples were weighed only when maximum accuracy was desired. The exact positioning of the sampling tube was facilitated by a device shown in figure 1. The holder was built onto a standard taper joint, which fitted the upper joint of the electrode vessel directly above the cell. A calibrated scale on the holder with a reference line on the upper end of the tube

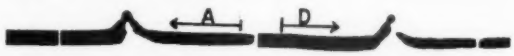

Fig. 2—An electrophoretic pattern of type O virus produced in vitro and purified by three cycles of differential centrifugation. The pattern was obtained after forty-six minutes at 7.1 volts/cm. in 0.1 ionic strength phosphate buffer, pH 7.5.

From the State Veterinary Institute for Virus Research, Lindholm, pr. Kalvehave, Denmark, and the U. S. Bureau of Animal Industry, Washington, D. C.

Author's present address is Naval Biological Laboratory, Naval Supply Center, Oakland, Calif.

The helpful suggestions and assistance of Dr. A. Randrup and Mr. E. Anderson are gratefully acknowledged as valuable contributions to this study.

* Manufactured by Specialized Instrument Co., Belmont, Calif.

** Manufactured by American Instruments Co., Silver Spring, Md.

made it possible to determine the position of the tube in the cell. This was particularly advantageous when the solutions were turbid or when

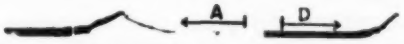

Fig. 3—An electrophoretic pattern of type O virus produced in vitro and purified by two cycles of differential centrifugation. The pattern was obtained after eighty-four minutes at 6.6 volts/cm. in 0.1 ionic strength veronal buffer, pH 8.6.

samples were desired from sections of the cell not visible on the viewing screen. In the Aminco unit, approximately 2 cm. of the cell was not projected on the viewing screen. This glass device, attached directly to the electrophoresis cell rather than to the frame, helped prevent vibration of the needle as it was lowered into position.

Appropriate tenfold dilutions, made in phosphate buffer pH 7.5, were injected into the footpads of guinea pigs. The titer of the crude virus preparations were between 10^3 and $10^{4.5}$ infective doses.

RESULTS

Figure 2 represents the electrophoretic patterns obtained with purified virus. Because the virus was most stable at pH 7.6, the first determinations were made in 0.1 ionic strength phosphate buffer, pH 7.5. However, figure 3 shows that similar patterns were obtained in 0.1 μ veronal buffer at pH 8.6.

Unfortunately, the main peak did not represent pure virus because similar patterns were obtained with blind cultures (fig. 4). Consequently, the major component of purified virus preparations must consist of normal heavy constituents of such tissue cultures. However, the results summarized in table 1 indicate that the electrophoretic mobility of the infective particles was very similar to that of the "visible" components in the purified preparations. Infectivity of virus purified by two or more cycles of differential centrifugation was rapidly lost at pH 7.0, but a less purified preparation was sufficiently stable at pH 7.0 to permit titration. Table 1 shows that the main component of such partially purified preparations had an electrophoretic mobility near that of the infective particles.

The virus was rapidly inactivated below 7.0, but it has been reported [7] that it will remain infective if the pH is rapidly lowered to 3.0. Therefore, attempts were made to determine the isoelectric point of the virus from measurements at pH 3.0 and pH 7.0. The electrophoretic pattern at pH 3.0 (fig. 5) shows two components in the partially purified virus preparations, but no virus could be detected in any of the samples after electrophoresis.

The electrophoretic mobilities of the optically demonstrable components in virus preparations at the various hydrogen ion concentrations are plotted in figure 6. Because there was no evidence that the infectivity was associated with either of the two main visible components at pH 3.0, the isoelectric point of the virus could not be estimated on the basis of figure 6.

DISCUSSION

Janssen's [1] electrophoretic study and identification of foot-and-mouth disease virus was based on comparisons between infectious lymph and normal serum. Since he observed a component in lymph which was not present in serum, he concluded

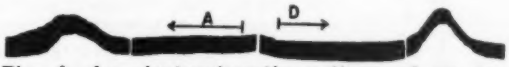

Fig. 4—An electrophoretic pattern of an extract of normal bovine tongue epithelium after three cycles of differential centrifugation comparable to that employed in the purification of the virus. The pattern was obtained after 103 minutes at 6.9 volts/cm. in 0.1 ionic strength phosphate buffer, pH 7.5.

that it was the foot-and-mouth disease virus. Unfortunately, Janssen did not make comparisons with extracts of normal epithelium from bovine tongues. By compar-

TABLE 1—The Observed Electrophoretic Mobility of Purified Foot-and-Mouth Disease Virus Preparations

Solvent	Ascending Mobility*		Descending Mobility*	
	Visible peak	Infectivity between	Visible peak	Infectivity between
pH 7.0, 0.1 μ phosphate buffer	−4.8	−4.6 and −5.3**	−4.4	−3.8 and −4.5**
pH 7.5, 0.1 μ phosphate buffer	−6.3	−5.9 and −6.8	−5.7	−4.5 and −5.6
pH 7.5, 0.06 μ veronal buffer	−6.2	−6.5 and −6.8	−5.6	−4.5 and −5.6
pH 8.6, 0.1 μ veronal buffer	−5.2	−5.0 and −6.1	−4.1	−3.8 and −4.4

* Mobility x 10^{-5} cm.^{2}/sec./volt; ** based on partially purified preparations.

ing figure 4 and figures 2 and 3, it may be seen that the electrophoretic pattern of purified heavy components from normal tissue was similar to the pattern produced by the purified virus. Furthermore, serologi-

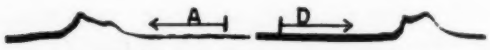

Fig. 5—An electrophoretic pattern of type O virus produced in vitro and purified by two cycles of differential centrifugation. The pattern was obtained after seventy minutes at 6.0 volts/cm. in 0.1 ionic strength phosphate buffer, pH 3.0.

cal studies [8] have indicated that the most highly purified virus preparations available in this laboratory still contained a large amount of impurities. However, the serological evidence must not be considered absolute proof, because it is possible that the virus particles may contain or be absorbed to elements characteristic of normal protein. Knight [9] has shown that the influenza virus particles produced either in mice or in embryonating eggs were serologically related to the host proteins.

In addition to the comparative studies, Janssen published an electrophoretic diagram of a virus preparation purified by ultracentrifugation and he implied that the virus was relatively pure. This component, identified as the foot-and-mouth disease virus by Janssen, probably was identical with the "visible" component observed in the purified preparation shown in figure 2.

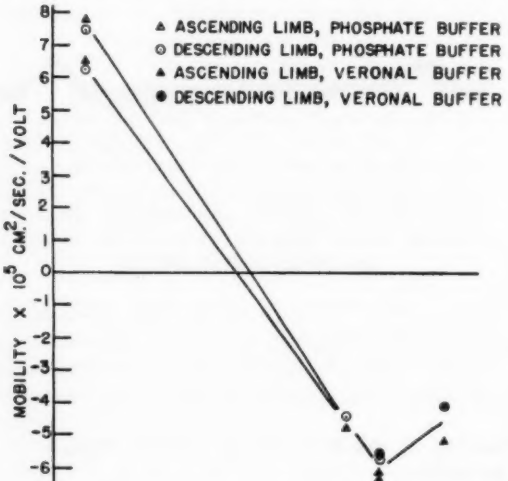

Fig. 6—The electrophoretic mobility of the main optically demonstrable components in purified preparations of foot-and-mouth disease virus at various hydrogen ion concentrations.

An interesting point that is apparent in figure 6 is that the electrophoretic mobility of the optically demonstrable component was greater at pH 7.5 than it was at pH 8.6. It has been suggested that this maximum mobility may be related to the maximum stability of infectivity because it has been reported [7] that the virus was most stable near pH 7.6. Pyl [7] also indicated that the minimum stability of foot-and-mouth disease virus was between pH 5.7 and pH 6.5, which might correspond with the isoelectric point of the "visible" components in purified preparations.

SUMMARY

A method for removing well-defined samples from a Tiselius-type electrophoresis cell was described.

It has been determined that the infective particles of foot-and-mouth disease virus and the optically demonstrable component in purified preparations have similar electrophoretic properties between pH 7.0 and pH 8.6. The mobility of the virus at pH 7.0, 7.5, and 8.6 was approximately —4.6, —6.0, and -4.8×10^{-5} cm.^{2}/sec./volt, respectively.

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Studies of the Sheep Mite, *Psorergates Ovis*

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THE FINDING of the sheep mite, *Psorergates ovis*, in a flock of fine-wool Merino sheep was reported in 1952 by Bell *et al.*[1] of the Ohio Agricultural Experiment Station. This was the first time it had been reported in America. Little is known of the incidence and biology of the genus, Psorergates, and the resulting economic loss. Consequently, this flock of Merino sheep was maintained for further study and experimentation. In order to accumulate data regarding this mite, it was decided to study its life cycle, develop a more satisfactory method for detection of the mite, study its transmission, and attempt to determine its economic importance to the sheep industry.

Three species of the genus, Psorergates, have been recorded in the literature. In 1883, Tyrrell[5] described *Psorergates simplex* found on a field mouse, and in 1889 Michael[6] described *Goniomerus musculinus* from a field vole, which was later classified by Oudemans[7] as *Psorergates musculinus*. In 1941, Carter[3] of Australia reported the finding of a new species of Psorergates on fine-wool Merino sheep. In 1941, Womersley[9] confirmed the classification of Carter's specimen and described the mite as a new species, *Psorergates ovis*.

IDENTIFICATION PROCEDURE

One of the problems associated with the study of *Psorergates ovis* was the finding and identification of the mite. The usual methods used by Carter[3] and Bell *et al.*[1] of obtaining skin scrapings and preparing slides for examination were not entirely satisfactory. Numerous variations of these methods were tried and a more satisfactory method was developed as follows.

The wool on the area to be examined was clipped and 2 *cc.* of a 2 per cent procaine solution was injected subcutaneously. A small amount of glycerine was applied to the clipped area. The skin was scraped with a knife blade, held approximately at a right angle to the skin surface as recommended by Benbrook,[2] until a small amount of blood appeared. An area approximately 1 in. square was scraped and the material taken to the laboratory for examination. Two types of mediums were used to prepare the slides for examination. These were Lamiman's modification of Berlese medium, as used by Carter,[3] and 10 per cent potassium hydroxide as recommended by Koutz.[4] A comparison of these mediums was made to determine their value as an aid in identifying *P. ovis*. Lamiman's modification of Berlese medium was found to be superior to 10 per cent potassium hydroxide in our work. It stains the mite so the various forms are more easily distinguished and identified. This medium is easy to prepare, has good keeping qualities, and permanent mounts may be made with it. Less eye strain was noted when this medium was used. It was observed that 10 per cent potassium hydroxide did not destroy the identifying characteristics, as has been reported by Koutz *et al.*[4,5] Mature *P. ovis* in skin scrapings remained in 10 per cent potassium hydroxide for twenty-four hours after digestion of the detritus without destruction of the identifying characteristics. The immature forms of the mite were rarely seen when this medium was used.

FLOCK SURVEY

The flock of sheep utilized in this survey consisted of 48 ewes 2 years or more of age, with their lambs; 25 ewes approximately 1 year old; and 11 mature rams. The ewes with their lambs were placed in one enclosure, the yearling ewes in a second, and the rams in a third, all within the same barn.

Skin scrapings were made from a total of 94 animals in this flock. Twelve of the 48 ewes with their lambs were found to be infested with *P. ovis;* 4 of the yearling ewes were also positive, but only 1 ram was found to harbor this mite (table 1). Of the 12 ewes found to be infested, 9 were 7 to 8 years old; 1 was 5 years old; 1 was 3 years old, and 1 was 2 years old.

Seven of these infested ewes had a total of 10 lambs approximately 1 month of age. Skin scrapings were made from these lambs, but no mites were found. At least 121 *P. ovis* in various stages were found in one scraping from the ram, more than from any other single scraping taken. A year-

TABLE 1—Percentage of Infestation with Psorergates Ovis in a Flock of Fine-Wool Merino Sheep

Type of sheep	Age	No. scraped	No. infested	Percentage of infestation
Ewes	2 yr. or more	48	12	25.00
Ewes	Approx. 1 yr.	25	4	16.00
Rams	2 yr. or more	11	1	9.09
Lambs	Approx. 1 mo.	10	0	0.00

From a thesis submitted by the author in partial fulfillment of the requirements for the degree of master of science of Ohio State University.

This is a coöperative project of the Department of Veterinary Parasitology, College of Veterinary Medicine, Ohio State University; the departments of Veterinary Science and Animal Science, Ohio Agricultural Experiment Station; the Ohio State Department of Agriculture; and the Bureau of Animal Industry, United States Department of Agriculture.

ling ewe and a 3-year-old ewe were also heavily parasitized. The mites were found in skin scrapings taken from various areas of the body.

Only part of the old ewes which showed taggy wool were infested with *P. ovis;* 2 of 3 with a heavy infestation did not show taggy wool. The taggy wool in the flock as a whole was less noticeable in the early part of April, 1953, than it was in November, 1952. None of the yearling ewes showed taggy wool, although 4 of the 25 were infested with the mite.

No scratching was noted after they had been sheared, although the 3 infested with *P. ovis* before shearing still carried the infestation thirty-two days after they were sheared.

After a survey of the flock, the ram was selected for special study since he showed the greatest infestation with *P. ovis.* Two additional skin scrapings were taken from him in March. At no time did he show signs of scratching, biting, or taggy wool. He had a more open type of fleece than some of the rams and was in good flesh.

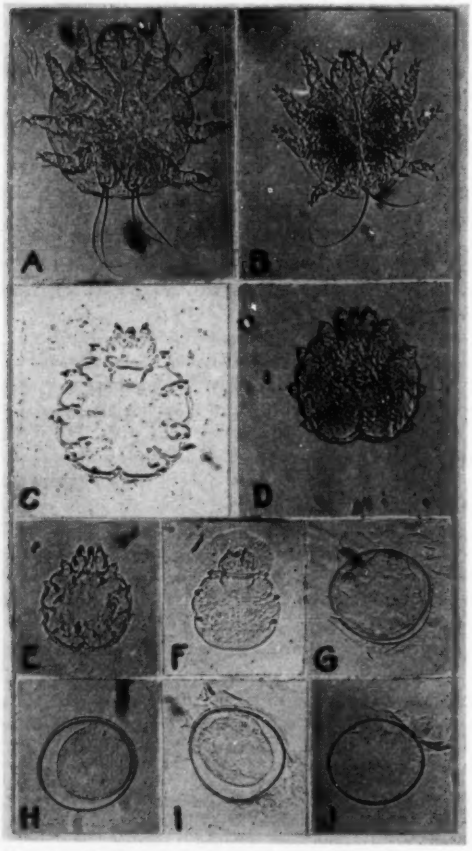

Fig. 1—Various phases of Psorergates ovis. A) adult female; (B) adult male; (C,D,E) various stages of nymph; (F) larval stage; (G,H,I,J) various stages of egg. x 120.

The yearling ewe with the greatest infestation of *P. ovis* was also used for this study. She was in good flesh and did not show itching or taggy wool. One scraping was taken and slides were made with Lamiman's modification of Berlese medium.

LIFE CYCLE

The complete life cycle of *P. ovis* is not known. Therefore, the morphology of the mite was studied in detail so that each stage of the life cycle could be identified.

A typical mite life cycle consists of an egg, a larva, a nymph, and adult stages. All these stages were found on the ram but only the egg, nymph, and adult female were found on the yearling ewe. This would indicate that *P. ovis* can complete its life cycle on an individual animal; however, more work must be accomplished before ruling out the possibility of a carrier host.

Figure 1 shows the various stages of *P. ovis* found on the ram and includes the adult male and female, and various stages of the egg, larva, and nymph.

TRANSMISSION OF PSORERGATES OVIS

An infested fine-wool Merino ewe and her lamb were placed in an enclosure in March to determine whether this mite could be transmitted by direct contact. Skin scrapings had shown this ewe to be positive for *P. ovis,* while skin scrapings made from her lamb were found to be negative. Two noninfested Merino ewes and a lamb were obtained in March from a different area of the state and were isolated for two weeks. Skin scrapings taken at approximately ten-day intervals during this period were found negative for all species of mites. They were then placed in the enclosure with the infested ewe and her noninfested lamb.

Skin scrapings of the test animals were negative during April, May, June, July, August, September, October, and November but on December 15, *P. ovis* were found on the 2 previously noninfested Merino ewes.

SUMMARY

1) An examination was conducted for the presence of infestation with *Psorergates ovis* in a flock of 94 sheep where this parasite had previously been found. There were 48 ewes over 2 years of age, 10 lambs approximately 1 month of age, 25 yearling ewes, and 11 rams. Twelve ewes, 4 yearling ewes, and 1 ram showed an infestation with *P. ovis.*

2) The egg, larva, nymph, and both adult male and female stages of *P. ovis* were found on the ram. The egg, nymph, and adult female stages only were found on 1 yearling ewe.

3) Lamiman's modification of Berlese

medium was superior to 10 per cent potassium hydroxide for finding *P. ovis*.

4) *Psorergates ovis* mites were not found on 2 previously noninfested Merino ewes until they had been kept in the same enclosure for approximately nine months with an infested Merino ewe.

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Cerebrospinal Fluid Pressure of Normal and Vitamin A Deficient Swine as Determined by a Lumbar Puncture Method

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DURING a series of experiments to determine the minimum carotene requirements of swine, it was necessary to develop a method of measuring the cerebrospinal fluid pressures of young pigs. Sykes and Moore [4] and Eveleth *et al.*[1] found in cattle and sheep, respectively, that avitaminosis A caused an increase in cerebrospinal fluid pressure.

This report describes the lumbar puncture method used for cerebrospinal fluid pressure measurement in swine and gives the values obtained by this method with normal and vitamin A deficient swine. An Ayer spinal fluid manometer was found to be satisfactory for recording the pressure and for withdrawal of fluid for analysis. This apparatus consists of two-glass manometer tubes graduated in millimeters, a special three-way stopcock, and a 20-gauge 3¼-in. rustless spinal needle with stylet. As the apparatus can be sterilized by chemical disinfectants, it is adaptable for field use.

It is necessary for the pig to be anesthetized while the reading is taken because movement by the pig can result in an increased pressure reading. An intraperitoneal injection of 5 per cent chloral hydrate at the rate of 2 ml. per pound of body weight proved to be a satisfactory anesthetic. The anesthetized animal is laid in lateral recumbency with the spinal column as nearly horizontal as possible. An extensive area in the lumbosacral region is shaved, washed with ether, and painted with tincture of iodine.

The puncture is usually made between the last lumbar vertebra and the sacrum. The needle may be inserted between any of the lumbar vertebrae, however. Insertion of the needle into the lumbosacral space is recommended because blood may occasionally be mixed with the cerebrospinal fluid, in which case a rapid puncture may be made higher up and fluid obtained which is free from blood. The lumbosacral space is located by drawing an imaginary line across the anterior border of the wings of the ilii and palpating approximately 2 to 3 in. posterior to this line and exactly on the median line. Location of this space is facilitated if an assistant is available to bend the back by moving the hind legs of the pig while the operator is palpating the area.

The puncture is made in the median line with the needle directed ventrally and anteriorly. The total depth of the puncture will vary with the age and degree of fatness of the animal. A slight "give" will be felt when the needle passes through the dura and arachnoid layers into the subarachnoid space. If the needle is inserted too deeply, the point will come against the body of the vertebra; in such instances, the needle must be withdrawn slowly. The stylet should be withdrawn when it is believed that the needle has entered the subarachnoid space. The first drops of fluid should appear after a few seconds. If no fluid appears, or if flow is too slow, a rotation of the needle or its slight withdrawal usually allows the fluid to flow freely. When the fluid is observed in the needle, the stopcock apparatus, with the graduated glass tube in a vertical position, is attached to the needle. The fluid will rise in the tube until it comes to rest, at which point the height of the fluid in millimeters is recorded. A small fluctuation in the level of the fluid to the extent of 4 to 6 mm. will result from expiration and inspiration of air in normal respiration. An absence of these pulsations may indicate lodging of tissue on the point of the needle.

When the initial pressure reading has been recorded, the assistant should apply moderate pressure to both sides of the neck to compress the jugular veins. The fluid should rise rapidly in the tube and subside slowly when the pressure on the

TABLE 1—Cerebrospinal Fluid Pressures of Apparently Normal Pigs

Pig (No.)	Age (weeks)	Cerebrospinal fluid pressures (mm.)
2	9	128
6	9	102
15	9	140
21	9	86
27	9	122
5	10	100
26	10	108
3	10	90
13	10	100
1A	20	80
2A	20	112
3A	20	80
4A	20	145
5A	20	138
Average pressure		109.5 mm.

From the Department of Veterinary Science (NS 133) (Sorensen and Kowalczyk) and the Department of Animal Husbandry (Hentges), University of Wisconsin, Madison. Published with the approval of the director of the Wisconsin Agricultural Experiment Station. Dr. Hentges is now assistant professor at the University of Florida, Gainesville, in the Department of Animal Husbandry and Nutrition.

neck is released. Failure of the fluid to rise rapidly may indicate either an obstruction in the needle or a subarachnoid block, resulting in an abnormal pressure reading.

Many different disturbances can cause fluctuations of the pressure. The action of the muscles, coughing, pressure of the hand on the abdomen, differences in position, mental excitement, and fear are a few of these influences. For these reasons, it is imperative that the pig be anesthetized.

The operation is rarely fatal if the anesthetic is properly administered and the spinal pressure is not lowered by removal of an excessive quantity of fluid or by drainage of fluid through the puncture canal.

The values obtained by this method on normal growing pigs varying in weight from 50 to 100 lb. are shown in table 1.

The average cerebrospinal fluid pressure for all the determinations was 109.5 mm., with a range of values from 80 to 145 mm.

Figure 1 shows the average cerebrospinal fluid pressures of growing pigs at three stages of an experiment in which all pigs, after being depleted of their vitamin A reserves, were allotted into four groups of 4 pigs each for supplementation with oral doses of crystalline carotene in cottonseed oil as follows: Lot 1 served as a control group and received no carotene supplement; lots 2, 3, and 4 received respectively 10, 25, and 40 μg. carotene per kilogram of body weight daily during the supplementation period. Two pigs in the control lot died during the experiment and the remaining 2 pigs exhibited severe deficiency symptoms and cerebrospinal fluid pressures of 212 and 220 mm. It is of interest that the cerebrospinal fluid pressures of pigs in lots 3 and 4 decreased during the supplementation period, but it did not return to the initial reading even though the animals were receiving adequate provitamin A to restore the vitamin A in their blood plasma to normal levels and to permit some storage of vitamin A in the liver. From these observations, it appears that cerebrospinal fluid pressures are of value for detecting the onset of a vitamin A deficiency but of less value for determining the state of vitamin A nutrition of animals which had previously been deficient in this vitamin.

SUMMARY

A method is described for determining the cerebrospinal fluid pressure of pigs by lumbar puncture.

The average cerebrospinal fluid pressure

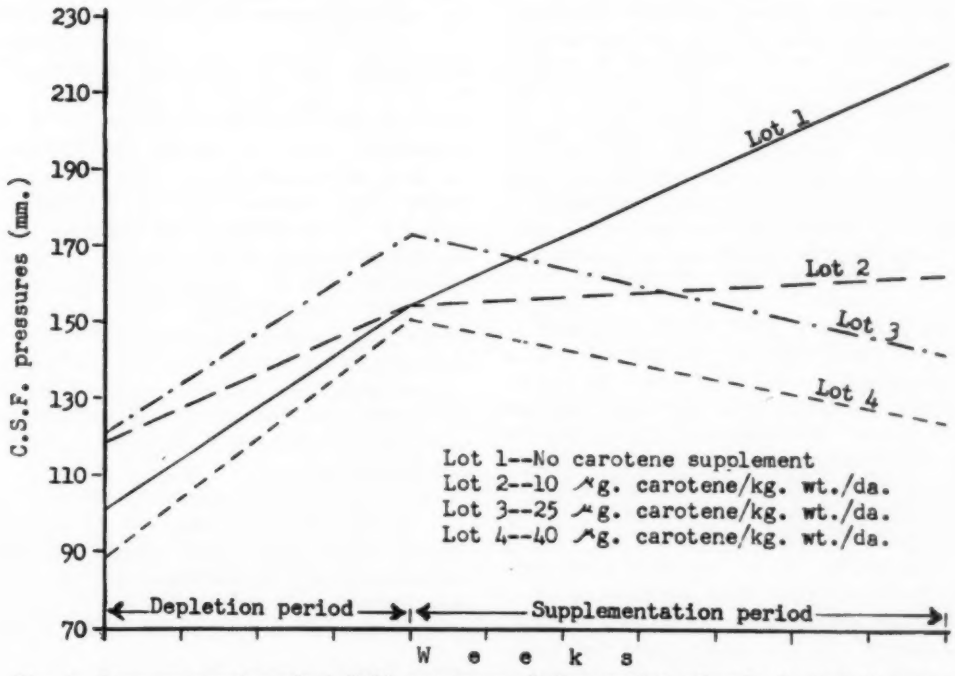

Fig. 1—Average cerebrospinal fluid pressures of pigs during vitamin A depletion and subsequent carotene supplementation.

of 14 apparently normal pigs was 109.5 mm. The values ranged from 80 mm. to 145 mm.

An increase in cerebrospinal fluid pressure up to 220 mm. was observed in pigs which were deficient in vitamin A. This method may be used to detect the onset of a vitamin A deficiency.

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A Study of Corneal Healing in the Dog

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THIS REPORT describes a limited experimental study of corneal healing in the dog. The primary purpose of the investigation has been to observe corneal healing when the usual vascular response to injury is interrupted. Since the use of *beta* rays to arrest corneal vascularization following injury has not been previously reported, it is believed that such observations will complement the recent reports about the similar effect of cortisone following experimental corneal injury.

This study has been stimulated by the clinical impression that corneal vascularity is often associated with delayed healing and permanent disfiguring of corneal tissue due to pigmentation. It was considered desirable that the role of vascularization in corneal healing be estimated. The ability of *beta* rays to destroy corneal blood vessels has become known to veterinarians. Thus, it provides an excellent method of influencing this phase of corneal inflammation and affords an opportunity for appraising the effect of vascularization in corneal healing.

There have been few, if any, reports in veterinary literature concerning the use of dogs in laboratory studies of keratitis. This investigation, therefore, was concerned with the problem of producing in dogs an experimental corneal wound that would cause a consistent inflammatory response and which would add to past information concerning the etiology of this pathology in the dog. The canine corneas that were not treated following experimental injury demonstrated the nature of spontaneous corneal healing. The clinical appearance and healing of ulcerative keratitis were correlated with the interval elapsing from the date of injury. This information, to the authors' knowledge, has not previously appeared in the veterinary literature.

Rabbits have been used almost exclusively in the experimental production of corneal ulcers. They have prominent corneas in which an intense inflammatory response is easily elicited. In addition, they may be conveniently destroyed during any particular phase of the experiment for histopathological study.

Most early attempts to produce corneal ulcers utilized application of local irritants, such as ethyl alcohol[1] or mustard gas.[2] Julianelle *et al.*,[3] in 1933, produced corneal ulcers in rabbits by the intracorneal injection of specific protein to which the animals had been previously sensitized. Robson[4] records that, as early as 1867, Langhaus described the production of experimental corneal lesions by inoculation of *Mycobacterium tuberculosis*. This was fifteen years before Koch discovered the etiological agent and established its causative relations with tuberculosis. Since then, numerous attempts have been made to produce corneal ulceration and neovasculogenesis by inoculation of the conjunctival sac and/or corneal stroma with other pathogenic microörganisms such as *Bacillus pyocyaneus*,[2] Pneumococcus spp.,[4] *Micrococcus aureus*,[2] and hemolytic streptococci.[2]

More recently, corneal lesions have been produced by the intracorneal inoculation of irritating substances such as sodium hydroxide[5, 6] and hydrochloric acid.[6] Cogan[7] obtained similar results by intracorneal injection of water. He suggested that neovasculogenesis occurred only when there was swelling of the corneal stroma adjacent to preexisting vessels. Alloxan was injected intracamerally by Ashton *et al.*[8] to opacify the cornea. Consistent corneal lesions have been obtained by Campbell and Michaelson[9] with electrocautery. Arey and Covode[10] used a whirling sandpaper disc to abrade the cornea. Schaeffer[11] used a special knife to make two parallel incisions partly through the cornea. The cornea was then stained and the space between the incisions abraded with a spatula.

These few references, selected from the numerous reports in the literature, have served as a basis for our attempts to produce a consistent corneal lesion in dogs. We wished to produce identical lesions with a similar vascular response, so that the manner of healing in each case could be anticipated. Marked deviations from the expected inflammation and healing could then be attributed to the treatment. Numerous methods were tried before a satisfactory procedure was developed. It was observed that many canine corneas are markedly resistant to injury. There was early, spontaneous repair of the mechani-

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cal and chemical wounds inflicted early in the experiment.

EXPERIMENTAL PROCEDURE

The dogs were anesthetized with thiopental sodium. An eyelid speculum was applied and the eyeballs were fixed by grasping the bulbar conjunctiva with two Allis tissue forceps. A Lancaster guarded sclerotome* (fig. 1) was used to make three corneal incisions forming the shape of an equilateral triangle. The blade was set to cut 0.7 mm. deep. The width of the blade, 6.0 mm., was used to measure the length of each side of the triangle. The distance from the base of the triangle to the dorsal limbus was an estimated 4.0 mm. (fig. 2). The corneal epithelium, Bowman's membrane, and part of the stroma in the center of the triangle were then abraded. Finally, twelve- to fourteen-day blood agar plate cultures of *Pseudomonas aeruginosa* were rubbed into the abraded surface.

Fig. 1—A Lancaster guarded sclerotome.

Pseudomonas organisms were used because of the clinical observation that persistent ocular infection in the dog is often due to this organism. Theodore and Feinstein [12] have also reported that Pseudomonas spp. are responsible for all recent human infections due to contaminated ophthalmic solutions. In addition, these authors noted that the organism produces the most serious type of corneal ulcer encountered in human ophthalmology. The particular strain of organisms used in this experiment was pathogenic for the guinea pig from which it was isolated. It was observed that a much greater inflammatory response occurred with old, highly pigmented cultures of Pseudomonas organisms than with young, actively growing forms.

The 14 healthy corneas of 9 dogs were injured as described in the first phase of the experiment. These corneas were not treated and the animals were observed daily until healing was completed. Both corneas of a second group of 12 dogs were abraded in a similar manner. The left corneas of this group were also left untreated to serve as controls while the right corneas were treated with a *beta* ray applicator emitting 41.3 roentgen equivalent *betas* per second. The treatments were usually initiated on the third day following injury. This was generally the day preceding the gross appearance of corneal vessels. Irradiation for three-minute periods was centered on the limbal area dorsal to the lesion. Usually, several days passed before the effects of irradiation could be appreciated. It was found that about three treatments, at three-day intervals, afforded the minimum radiation necessary to almost inhibit vascularity. No supplemental therapy was administered to the irradiated corneas, and inspection of the healing progress was made daily.

The dogs used in this investigation were healthy adults from 2 to 8 years old and of mixed breeds. Animals with evidence of gross parasitism were given specific anthelmintic treatment. They were not affected with gross ocular or perioecular diseases, nor did any of the dogs have senile cataracts

TABLE 1—Observations on 9 Dogs with Untreated, Experimentally Injured Corneas

Experimental dog	No. of corneas abraded	Early corneal changes (24 to 48 hr.)	Later corneal changes	Time required for healing
1	1	Abraded area opaque	Neovascularization 5th day; keratocele, 8th day	51 days
2	2	Extensive edema; vessels visible at limbus	Vessels about lesion on 5th and 7th day, respectively	17 and 18 days, respectively
3	1	Abraded area opaque; cornea edematous	New vessels appear on 5th day	13 days
4	1	Abraded area opaque; cornea edematous	New vessels appear on 4th day	16 days
5	1	Abraded area opaque; mild edema	Numerous vessels at limbus on 5th day	17 days
6	2	Abraded area opaque; vessels at limbus	Vessels surrounding lesion on 6th day	10 and 15 days, respectively
7	2	Abraded area clouded; opaque on 2nd day	Vessels at limbus on 4th day; surround lesion on 7th day	13 and 17 days, respectively
8	2	Abraded area clouded	Vessels at limbus on 4th day; surround lesion on 7th day	24 and 31 days, respectively; 1 cornea pigmented
9	2	Corneas clouded; abraded areas opaque	Vessels appear on 3rd and 4th days; surround lesions on 8th day	21 and 24 days, respectively
Total, 9	14			Ave., 20.5 days

* Storz Instrument Co., St. Louis, Mo.

or visible corneal scars. They were confined to clean, dry cages and fed a ration of two-thirds commercial dog cereal foods and one-third cooked horse meat. During the course of the experiment, several dogs which eliminated semiformed stools were found to be infected with coccidia and were treated daily with sulfathalidine tablets until remission of symptoms, or for one to two weeks.

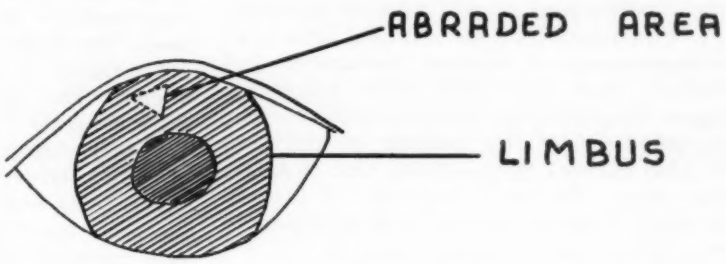

Fig. 2—The eyeball showing the relationship of the abraded area to the limbus.

EXPERIMENTAL RESULTS

A consistent inflammatory response was observed in the corneas that were eroded and then inoculated with a twelve- to fourteen-day Pseudomonas culture. The regular occurrence of corneal and conjunctival inflammation in the 14 corneas of the 9 dogs established the described technique as being capable of producing predictable pathology in canine eyes. The untreated left corneas of the 12 dogs afforded additional evidence concerning the nature of the keratitis resulting from this type of injury.

Conjunctivitis, following corneal injury, was observed within twenty-four hours. Swelling and hyperemia were the conjunctival changes that were noticed promptly. The bulbar conjunctiva became markedly congested, the area of deepest congestion being immediately adjacent to the corneal lesion. The conjunctival alterations gradually diminished as the lesions healed. The conjunctivas usually resumed their normal appearance about a week prior to final corneal healing. Persistent changes affecting this tissue were not observed.

The earliest corneal changes affected the abraded site. This area became opaque within twenty-four hours. The appearance of a band of fine blood vessels, located immediately within the cornea and above the abraded site, was usually noticed within four days following infliction of the experimental injury. When first detected, these vessels measured from 0.5 to 1.0 mm. in length. Neovasculogenesis proceeded at a variable rate, but usually within seven days the newly formed corneal vessels had reached the abraded site. Clouding of the cornea adjacent to the vascular area occurred in most cases. Anastomosis of new vessels was noticed at the injured site.

TABLE 2—Observations on 12 Dogs with Injured Corneas—One Irritated,* One Untreated**

Experimental dog	Involved cornea	Degree of corneal inflammation	Time required for healing
10	Left eye	Similar to untreated dogs**	21 days
	Right eye	Considerably less vascular	27 days; pigmented
11	Left eye	Similar to untreated dogs	25 days
	Right eye	No gross vascularization	9 days
12	Left eye	Marked inflammation	17 days
	Right eye	Slight inflammation	24 days
13	Left eye	Mild	9 days
	Right eye	Mild	13 days
14	Left eye	Marked	24 days
	Right eye	Marked; developed slower	27 days
15	Left eye	Marked	22 days
	Right eye	Slight; less vascular	26 days; pigmented
16	Left eye	Marked	22 days
	Right eye	Marked; developed slower	27 days
17	Left eye	Marked	23 days
	Right eye	Less vascular	25 days
18	Left eye	Not used	
	Right eye	Mild	14 days
19	Left eye	Mild	20 days
	Right eye	Mild	22 days
20	Left eye	Marked	23 days
	Right eye	Very slight vascularity	17 days
21	Left eye	Marked	13 days
	Right eye	Very slight vascularity	13 days
Total, 12	11 left eyes	Untreated	Ave., 19.9 days
	12 right eyes	Treated	Ave., 20.3 days

* The right corneas were treated with *beta* radiation three to five times each at intervals of 2 to 5 days.

** The left corneas were similarly abraded and left untreated (*see* table 1).

These vessels usually invaded and partially surrounded the excavated area. Complete vascularization of the lesion rarely occurred.

The criteria used to measure healing of the experimental corneal wounds included: (1) cicatrization of the abraded area; (2) disappearance of corneal vascularity and edema; and (3) relief from subjective signs, as pain and photosensitivity. The period required for healing of the nontreated corneas ranged from nine to fiifty-one days. The great majority of injured corneas, however, healed within fifteen to twenty-five days (tables 1 and 2).

It was observed that a cicatrization persisted at the abraded site of all injured corneas after the inflammatory changes had subsided. Areas of brownish pigmentation developed in three corneas, two of which had been treated with *beta* radiation. The pigmentation persisted in each instance after the lesion was considered to be healed.

The 12 corneas that were treated with *beta* radiation were compared with the nontreated corneas in degree of inflammatory response to injury and time required to heal. The conjunctival alterations that occurred within the irradiated eyes were similar to those in the nontreated group. Gross observations of the irradiated corneas revealed that neovasculogenesis was markedly impaired, new vessels appearing later and developing more slowly than in the nonirradiated group. It was not possible to prevent the occurrence of all neovasculogenesis with *beta* radiation, as employed in this experiment. The degree of corneal vascularity was considerably less in all irradiated eyes than in the nontreated ones.

The abraded sites of the irradiated corneas retained their excavated appearance for three to seven days longer than did the nontreated lesions. Healing for eight of the 12 irradiated corneas required from two to seven days longer than the untreated corneas in the same dog. However, two of the irradiated corneas healed several days earlier than those which were not treated (table 2).

These results indicate that corneal neovasculogenesis, as observed grossly, is a functional part of corneal healing which, if interfered with, caused a delay in the healing of a majority of cases.

DISCUSSION

The failures that marked the early attempts to produce a consistent keratitis in this experiment indicate that the etiology of ulcerative keratitis in the dog may be composed of a combination of factors. It is likely that infection of a corneal laceration or abrasion is a common etiological sequence. The role of malnutrition in ulcerative keratitis of dogs was not estimated in any way during this experiment. It was observed, however, that a few thin dogs, whose coats were dry and whose skins were inelastic, were no more susceptible to corneal injuries than were the majority of dogs which appeared to be in a good nutritional state.

An interesting feature of corneal reaction to injury occurred in several dogs during this experiment. The degree of inflammatory response was invariably greater and its onset more rapid when normal appearing corneas were reinjured following their apparent recovery. An increased response to subsequent abrasions will also likely occur in clinical cases of ulcerative keratitis.

The results of this experiment have several clinical implications. It was our experience that ulcerative keratitis was readily produced only when infectious organisms were introduced into an abraded surface. It seems probable that many cases of canine ulcerative keratitis represent an infectious and traumatic etiology. The marked pathogenicity of *Pseudomonas aeruginosa* for the canine cornea has been shown in this experiment. It might also be concluded that impairment of the initial vascular reaction to corneal injury will likely prolong the healing period.

SUMMARY

1) It has been demonstrated that a predictable inflammatory reaction can be consistently elicited in the canine cornea by a measured abrasion and infection.

2) It was observed that the rate of healing was delayed in a majority of canine corneas whose vascular response to experimental injury was interrupted with *beta* radiation.

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The Duct System of the Canine Pancreas

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IN RECENT YEARS, veterinarians have exhibited an increasing interest in canine pancreatitis. In reviewing the medical literature on this subject, it was found that *(a)* the dog is the animal of choice in producing experimental pancreatitis; *(b)* the methods most frequently employed for the production of pancreatitis are ligation and injections of various mediums into the pancreatic duct; *(c)* valuable information on spontaneous pancreatitis in the dog can be obtained from these experiments. Although the significance of the pancreatic ducts in the pathogenesis of pancreatitis is readily seen, very little is known about their normal anatomy. It appears that the presence of anatomical variations, particularly in regard to the number of ducts and the occurrence of interductal anastomoses, account for some of the discrepancies in medical texts. The purpose of this paper is to demonstrate the system of ducts in the canine pancreas by means of radiopaque mediums, a technique not previously utilized for this purpose.

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MATERIALS AND METHODS

Fifty dogs of both sexes, various breeds, and all ages constitute the basis for this work. The majority of dogs were healthy, although a few suffered various diseases unrelated to the pancreas. The animals were autopsied and the duodenum with adjacent pancreas were removed as soon as possible after euthanasia or death. The canine pancreas is a boomerang-shaped organ, with its head, duodenal, or right limb lying in the mesoduodenum, and the tail, transversal, or left limb situated in the omentum dorsal to the transverse colon (fig. 1, 2, 3, and 6).

The duct system of the canine pancreas is quite different from that of the human being. In dogs, the main duct is the duct of Santorini, opening into the duodenum at the minor duodenal papilla. The duct of Wirsung, which joins the bile duct at the ampulla of Vater and forms the major duodenal papilla in man, is small and not always present in the dog. A true ampulla of Vater is not formed in the canine species.

Demonstration of the duct system by means of dissection is difficult because of the membrane-thin walls of the ducts. In our experience dissection, even after injection of India ink, proved impractical. A method of injecting vinylite (a plastic compound which solidifies rapidly) into the main duct, followed by digestion of the pancreatic tissue in a strong acid solution, demonstrated the ducts and their anastomoses, but this method was

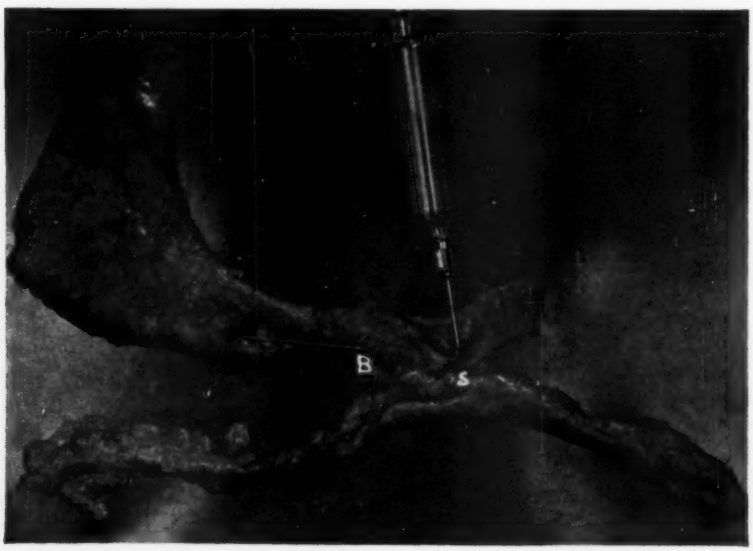

Fig. 1—Stomach and anterior part of duodenum with adjacent pancreas. The probe is inserted into the bile duct (B) and the syringe with needle in the main pancreatic duct (S) (duct of Santorini).

found to be inferior to the radiographic technique. By the latter method, radiographs are obtained that are easily recorded and filed, which is difficult with the fragile plastic casts. Secondly, the radiographs illustrate perfectly the relationship between the ducts and the contour of the pancreas, and between the ducts and the duodenum with the opening of the bile duct.

The pancreas was arranged on a sheet of cellulose acetate* in a position simulating its normal position in the dog. A probe was inserted into the bile duct so that the end of the probe indicated the opening of the bile duct into the duodenum on the major duodenal papilla. A blunt needle was inserted into the minor duodenal papilla and securely tied. Needles from 23- to 16-gauge were used, depending on the size of the opening in the minor duodenal papilla.

Several opaque mediums were tried. Iodopyracet (diodrast 35 %) gave good visualization of the duct system but tended to diffuse into the surrounding tissue almost as soon as injected. Medopaque-H showed a similar tendency. Iodized oil (lipiodol lafay) did not diffuse into the surrounding tissue but was found too viscous to be introduced into a duct system where fine shunts existed. Finally ethyl iodophenylundecylate (pantopaque) was selected as most suitable for this work. It was sufficiently fluid to be introduced into the finest duct systems encountered and did not diffuse into the surrounding tissue in the time required for the injection and for the making of radiographs. The amount of opaque medium necessary to outline the duct system varied with the size of the pancreas from 0.5 to 1.0 cc.

The injection was made with x-ray fluoroscopic control. The pancreas on the sheet of x-ray film was placed on the x-ray table. A sheet of lead rubber was placed so that the hands of the radiologist operating the syringe were protected from the x-ray source under the table. Lead rubber aprons were worn by the radiologist and those observing the injection. The fluoroscopic shutters were closed down to limit the illuminated screen area to approximately 10 in. by 2 in. By these precautions, it was possible to employ fluoroscopic control while making the injection with the minimum of radiation hazard.

* X-ray film from which the emulsions have been washed (x-ray film base).

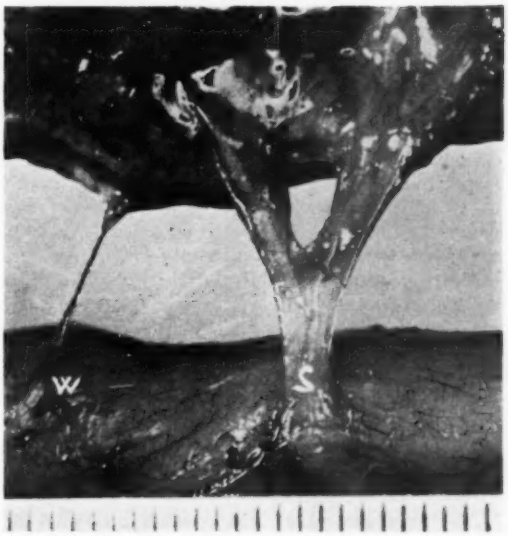

Fig. 2—Duodenum with pancreas above; to the right, the main pancreatic duct (S) branching, and to the left, the small pancreatic duct (W) (duct of Wirsung). The scale at the bottom indicates millimeter divisions.

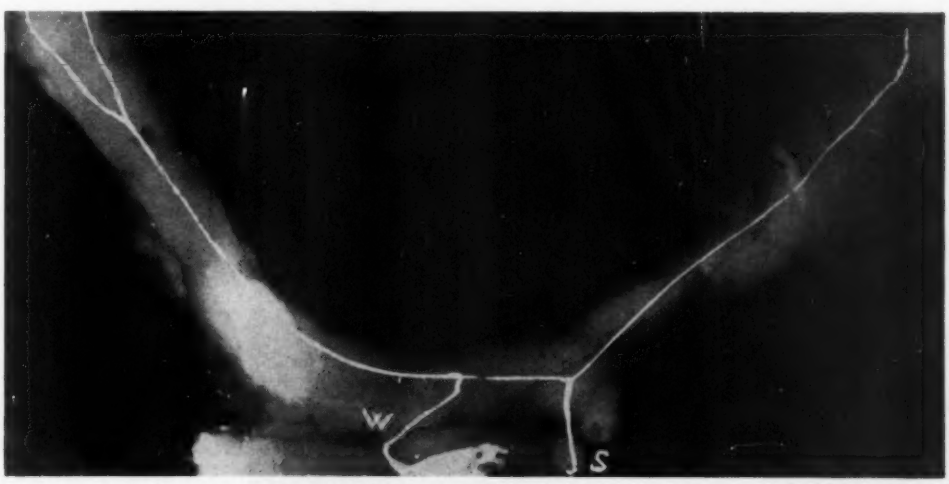

Fig. 3—A radiograph after injection of dye, illustrating a simple arrangement of the two ducts, type 1. Some dye has flowed into the duodenum via the duct of Wirsung (W).

The ease with which the radiopaque medium could be introduced into the pancreatic ducts appeared to depend on the time elapsed following the death of the animal and the arrangement of the duct system. When the pancreas was removed and an attempt made to introduce the opaque medium from zero to five hours after death, it was found that air was often trapped in the ducts and obstructed the flow. Care was necessary to avoid rupturing the ducts with excessive pressure while applying sufficient pressure to introduce the opaque medium into the various radicles of the system. It was found useful to make a small incision across the extremity of the limbs of the pancreas to assist the escape of any trapped air. In general, injections performed from five to twelve hours after death presented no special problems apart from those attributed to duct formation which are discussed below. Caution was necessary to insure that the entire duct system was filled.

The arrangement of ducts which presented the greatest difficulty in introducing the opaque medium was that of type 3 (fig. 8), where two ducts emerged directly at the two papillae, the only connection inside the pancreas being an extremely fine shunt. To outline the duct not directly connected to the needle, the opaque medium had to be eased through the fine shunt. To facilitate this, the handle was cracked from the plunger of a 20-cc. syringe so that mercury or lead shot could be introduced. With this device in an upright position, the opaque medium could be introduced into the finest ducts. The manual application of a light steady pressure was found very exhausting and often led to spoiled specimens.

RADIOGRAPHIC TECHNIQUE

Adequate screen brilliance for fluoroscopy was obtained by using 55 kv. p. at 3 ma.; for radiography, 26 to 27 kv. p. at 250 ma./sec., working at a focal film distance of 36 in. Ilford's ilfex, a fine grain nonscreen type of x-ray film, was used.

RADIOGRAPHIC FINDINGS

For convenience, we have divided the duct systems of the pancreas into five main types.

Type 1.—Twenty-three of 50 pancreas (46 %) had two main ducts, one from each limb coming together at a "Y" junction in the pancreas, with the tail of the Y terminating at the minor duodenal papilla. An additional duct emerges from the main duct in the left limb of the pancreas and enters the duodenum at, or very near, the major duodenal papilla. This pancreatic duct corresponds to the duct of Wirsung in man and it frequently follows a most indirect and tortuous route (fig. 4). The distance between the outlets of the two ducts into the duodenum varied from 8 to 54 mm., with an average distance of 28.

Type 2.—Eleven pancreas (22 %) were similar in general arrangement to those in type 1 with the exception that the duct forming the anastomosis emerged from the right limb of the pancreas, followed a route approximately parallel to the main duct of the limb before crossing the main duct of the left limb and emerging at, or near, the major duodenal papilla (fig. 6, 7). The distance between the outlets into the duodenum averaged 27 mm., with a greatest distance of 40 mm. and a shortest distance of 15 mm.

Type 3.—Eight pancreas (16 %) had a main duct which emerged from each limb of the pancreas and proceeded directly to the papilla opposite to the limb in which it originated (fig. 8). That is, the duct from the right limb emerged at the major duodenal papilla; the duct from the left limb

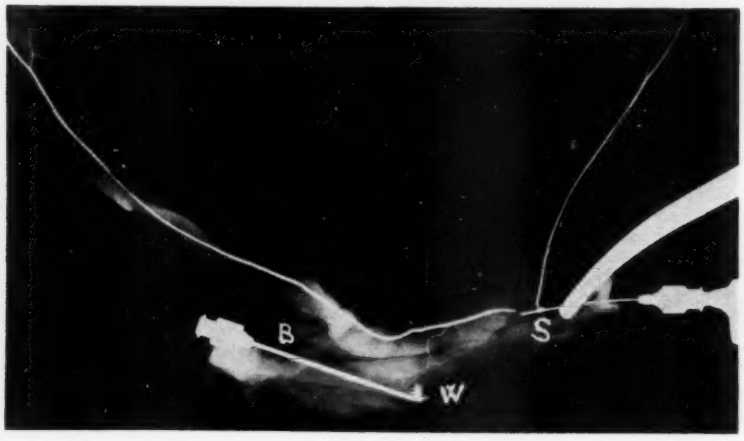

Fig. 4—The radiograph shows a pancreas after injection of radiopaque medium into the main pancreatic duct S. The other needle is inserted into the bile duct (B); its end indicates the ampulla of Vater, which contains some dye flowing back via the small pancreatic duct W, type 1.

crossed the duct from the right limb and emerged at the minor duodenal papilla. In addition, there is a very fine and often tortuous shunt connecting the two ducts. The distance between the outlets into the duodenum varied from 9 to 35 mm., with an average of 24 mm.

Type 4.—Four pancreas (8 %) showed the Y formation of the ducts but no other duct leading to the duodenum or the bile duct could be demonstrated (fig. 9). In two of the pancreas, an anastomosis existed between the two limbs inside the pancreas.

Type 5.—Four pancreas (8 %) were found with three duodenal orifices. Three of these pancreas were discovered to have a duct system which combined the arrangements found in types 1 and 2. The anastomosing duct, which originated in the duct of the right limb, emerged at or near the major duodenal papilla. The small duct which emerged from the duct of the left limb emerged some distance anteriorally. Another pancreas was found to have two additional small ducts leading into the duodenum, one on either side of the minor duodenal papilla. The one from the right limb emerged 16 mm. posteriorally, and that from the left limb 11 mm. anteriorally to the minor duodenal papilla.

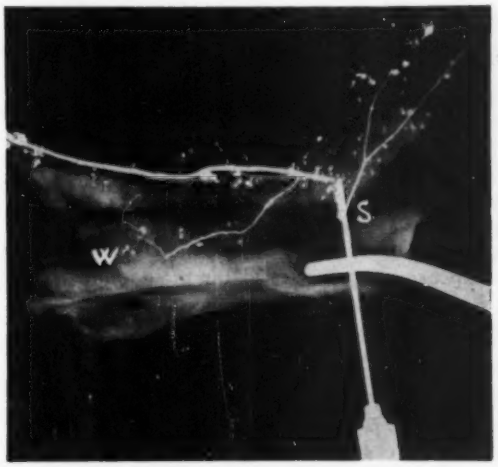

Fig. 5—An example of a pancreas in which the right main branch leaves the left branch within the wall of the duodenum. The duct of Wirsung (W) can be clearly visualized, type 1.

The minor duodenal papilla was used as the point of origin for all measurements as this could be positively identified on all the radiographs. The radiographs show that the distance from the pyloric sphincter to the major duodenal papilla varies from 3 to 7 cm. with an average of 4.8 cm.

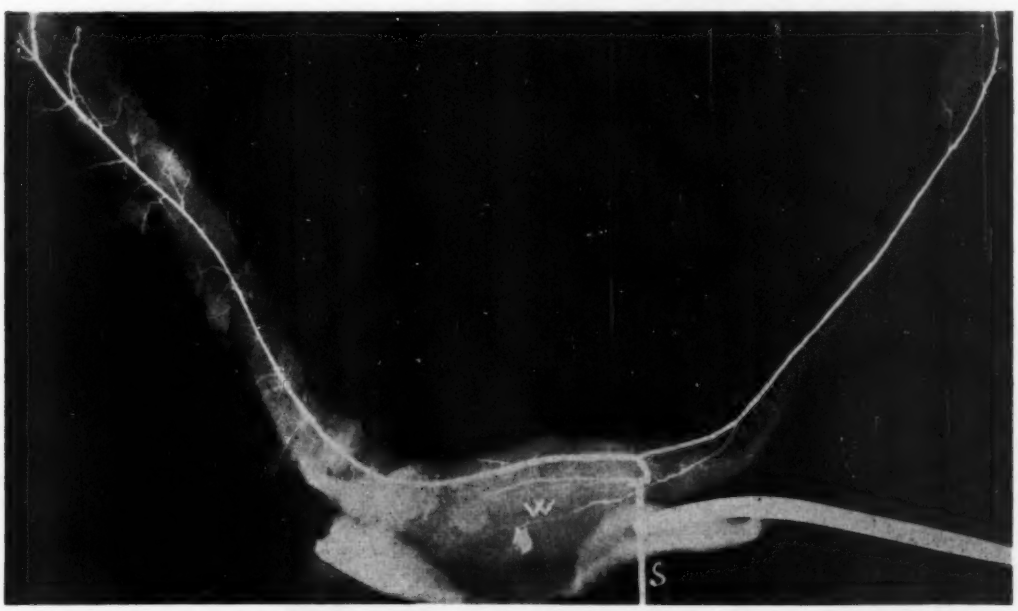

Fig. 6—Pancreas showing many of the smaller branches filled with dye. The small duct (W) is seen from the right main branch to the ampulla of Vater, type 2. Unusual is the finding of a duplication of the left main branch.

Fig. 7—A pancreas in which there is a dilatation at the junction of the two main branches. The duct of Wirsung (W) comes from the right main branch and goes to the duodenum, type 2.

DISCUSSION

This investigation showed the existence of five types of duct systems. Types 1, 2, and 3 can be grouped together as they have essentially the same features from a pathological aspect. The pancreas in the three groups (a total of 84 %) have two separate openings to the duodenum and in all cases the two ducts had interductal anastomoses within the pancreas. This means that obstruction of one of these two ducts (either experimentally by ligation or spontaneously by calculi, tumors, parasites, or an inflammatory process) will not cause any retention of secretion with the subsequent characteristic symptoms and lesions. The other still patent duct will take over the function and undergo hypertrophy and dilatation. The pancreas in group 4 (8 % of the dogs) showed no evidence of a functional second duct leading to the duodenum. Blockage of this duct would result in extensive lesions of the pancreas. The changes may be either a chronic acinar atrophy or an acute hemorrhagic necrosis, depending on the nature and the amount of pancreatic juice secreted at time of the obstruction. In group 5 (8 % of the dogs), three functional openings to the duodenum were found. The presence of this type in the dog population is probably one of the reasons for the conflicting results obtained in experimental ligation of the duct of Santorini and the duct of Wirsung in dogs. In the reports on spontaneous pancreatitis in man, much emphasis has been placed on etiological significance of bile entering the pancreatic duct system. From this study, it is seen that the theory that bile reflux produces spontaneous pancreatitis is not generally valid in the dog because of differences in the anatomy of the ducts. The human and feline species,[5,7] however, have a similar relationship of the bile duct and the duct of Wirsung. The two ducts join and form the ampulla of Vater, which opens at the major duodenal papilla. In the cat, the duct of Santorini is either absent or very small.

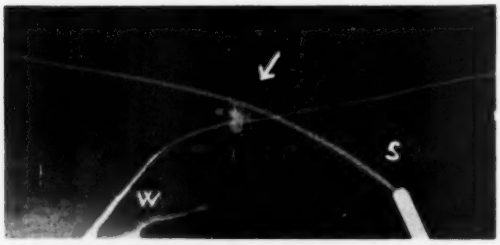

Fig. 8—A pancreas with a crossing of the main branches and formation of a peculiar fine shunt between them (see arrow). The dye is injected in the duct of Santorini (S) at the minor duodenal papilla and goes into the left limb. Dye is passing via the fine shunt into the right limb and back to the duodenum via the duct of Wirsung (W), type 3.

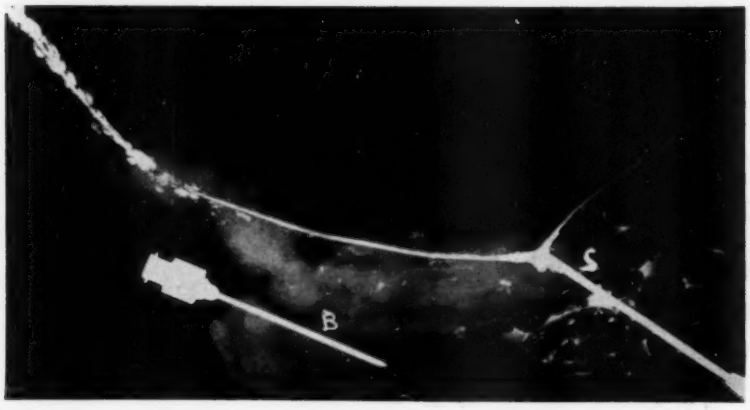

Fig. 9—A pancreas demonstrating the absence of the duct of Wirsung. A needle is inserted in the bile duct (B) for orientation, type 4.

SUMMARY

The pancreatic duct system in 50 dogs was injected with radiopaque mediums in order to visualize radiographically *(a)* the pattern of the ramifications of the duct; *(b)* the presence of interductal anastomoses; and *(c)* the number of openings into the duodenum. The common type (84 %) have two duct openings in duodenum, with interductal anastomosis present in the pancreas. Eight per cent had only one duct patent (the duct of Santorini) and another 8 per cent had three duct openings to the duodenum.

The bearing of the duct anatomy on the pathogenesis of pancreatitis is briefly discussed in the human and canine species.

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Experimental Treatment of *Trichuris Vulpis* Infections

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THE PATHOGENICITY of *Trichuris vulpis* is regarded by some practitioners as being of little importance; however, evidence has been accumulated to the contrary.[4, 9]

It is quite apparent in human whipworm infestations *(Trichocephalus trichiura)* that diarrhea or dysentery, frequently followed by visible prolapse of the rectum, often accompany heavy whipworm populations.[6] Other symptoms that have been attributed to the parasite are abdominal pain, loss of weight, poor appetite, dizziness, and weakness.[2] *Trichuris vulpis* has also been suspected of inciting chronic cecitis by providing portals of entry for bacteria.[11] The authors have had occasion to observe autopsies on dogs that were in extremely poor condition in which the walls of the cecums were highly inflamed and near the point of rupture. There would be unbelievably large numbers of whipworms within the lumen of these cecums.

The extent of damage produced by moderate infestations of whipworms has not been definitely established, but it appears that a satisfactory means of removal would still be desirable.

Many drugs and modes of administration have been employed in the treatment of dogs with whipworm infestations, though little improvement has been experienced since 1931. At that time it was stated that "the successful medicinal treatment of whipworm infestation in dogs is a problem, which thus far has defied all attempts at solution."[10]

The major difficulty encountered in the treatment of trichuriasis is thought to be the habitat of the parasite. Drugs administered orally could well pass by, instead of through, the ileocecal valve.[5] Lamson and his associates[7] demonstrated the possibilities of hexylresorcinol in the oral treatment of whipworm infestations, reporting the drug as being 50 per cent effective in a total study of 2,300 human cases. A few years following the work of Lamson *et al.*, Horning[5] reported favorable results with crystalline hexylresorcinol as an oral therapeutic agent for trichuriasis in 25 cases.

In a recent study of crystalline hexylresorcinol as an anthelmintic,[3] the drug was administered orally for the treatment of ascariasis and ancylostomiasis in dogs. Eight incidental whipworm cases were encountered in this work; of a total of 73 worms (average of 9.1 per dog), no worms at all were expelled by treatment. In this preliminary work, the oral administration of hexylresorcinol proved to be entirely unsatisfactory in the treatment of whipworm infestations. The authors[3] were unable to confirm the observations of Horning, though it should be pointed out that a different experimental procedure and analysis of results were employed.

Recent work in the treatment of trichocephaliasis in man by rectal infusion has indicated possibilities for hexylresorcinol in canine therapy for trichuriasis. Jung and Beaver[6] acquired excellent results with this method of administration using a 0.2 per cent suspension of hexylresorcinol. Basnuevo *et al.*[1] reported complete eradication of the parasites with a 1:300 suspension of hexylresorcinol given as a retention enema to 20 human patients.

In view of the generally poor results with hexylresorcinol in the oral treatment for whipworm infestations of the dog, and the recent favorable reports on rectal infusion in human beings, experiments were conducted in an attempt to determine the efficacy of the drug when administered as an enema to infected canine subjects.

MATERIALS AND METHODS

Parasitized animals were selected by egg-flotation tests (saturated sodium nitrate) and, in so far as was practical, the method of administration employed by Jung and Beaver[6] in human beings was adapted to 20 canine subjects. The time required for pretreatment cleansing enemas probably precludes their use by most practitioners of veterinary medicine. Therefore, this procedure was omitted in an effort to duplicate field conditions. A 0.2 per cent hexylresorcinol suspension was employed, 500 ml. being injected into either the colon or rectum. "Retention" being impractical in canine subjects, pressure was employed in an effort to force the medicinal agent into the lumen of the cecum. A great portion of the suspension, however, was ejected immediately after withdrawal of the enema apparatus.

The enemas were manipulated in two ways. On 10 of the subjects (series A, table 1) a flexible rubber catheter was passed with moderate pressure for varying distances into the colon, the suspension being forced into the region of the cecum by

TABLE 1—The Use of 0.2 per Cent Suspension of Hexylresorcinol in the Treatment of Trichuriasis Caused by Trichuris Vulpis

Series	No. animals	Worms (total)	Ave. No. worms	Removed (total)	Removed (%)
A	10	213	21.3	0	0.0
B	10	268	26.8	6	2.0
C	4	86	21.5	7	8.0

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a handbulb designed with a valve to prevent back flow. A second group of 10 (series B, table 1) received the same amount of hexylresorcinol suspension into the rectum by way of a pressure enema employing a device which fitted snugly into the anus.

TABLE 2—The Use of a 0.2 per Cent Suspension of Hexylresorcinol in the Treatment of Hookworm Infection

Series	No. animals	Worms (total)	Ave. No. worms	Removed (total)	Removed (%)
A	9	377	41.8	15	3.0
B	8	382	47.7	8	2.0
C	4	116	29.0	0	0.0

Theobald is quoted by Lewitus [8] as reporting the successful removal of whipworms in dogs by opening the abdominal cavity and injecting a suspension of santonin directly into the cecum. This procedure was employed on a third group of 4 animals (series C, table 1), a 0.2 per cent suspension of hexylresorcinol being injected, using a 28-gauge needle, into the distal end of the cecum. Moderate distention was obtained and the suspension held within the lumen by occluding the orifice for two minutes. The abdominal incision was then sutured and the animals allowed to recover from anesthesia.

In view of the results obtained in previous work [3] employing the oral administration of hexylresorcinol, the incidental hookworm and ascaris infestations were tabulated and the efficiency of the drug, when administered by enema, calculated (tables 2 and 3).

Immediately following treatment in each series of experimental animals (A, B, and C), the subjects were placed in individual cages. The cages were equipped with a special collecting pan to facilitate the preservation of all fecal matter passed. For forty-eight hours following treatment, all droppings were collected at twelve- to 24-hour intervals. The method for an accurate determination of percentage of effectiveness has been described,[3] the same procedure being duplicated for this study.

RESULTS AND DISCUSSION

It is apparent that hexylresorcinol, administered as an enema in accordance with the outlined procedures, would hardly be considered as fulfilling the properties of an effective anthelmintic.

Trichuris Vulpis.—It is demonstrated from the work presented that a 0.2 per cent suspension of hexylresorcinol administered as a pressure enema is entirely ineffective in the expulsion of *Trichuris vulpis* from dogs. These results appear to be in agreement with the observations of Underwood and his associates,[10] who concluded from their studies with tetrachlorethylene, oil of chenopodium, and ethylidene chloride that, "in view of the apparent difficulty in perfecting a technic for the intracaecal injection of anthelmintics, it would appear that this method is not of practical application in the treatment of whipworm infestations in dogs."

TABLE 3—The Use of a 0.2 per Cent Suspension of Hexylresorcinol as an Ascaricide

Series	No. animals	Worms (total)	Ave. No. worms	Removed (total)	Removed (%)
A	2	7	3.5	0	0
B	1	1	1.0	0	0
C	0	0	0.0	0	0

Ancylostoma Species.—The effectiveness of the colonic administration of a 0.2 per cent hexylresorcinol suspension failed to approach that obtained when the drug is administered orally.[3]

Ascariasis.—From the very limited data available in this study, it is difficult to evaluate the efficacy of hexylresorcinol administered by enema as an ascaricide.

SUMMARY

A brief account of past efforts toward the treatment of whipworm infestations has been presented. The results of this study offer little improvement toward a means of expelling the parasite. Incidental infestations of both hookworms and ascaris have been recorded, and it is shown that hexylresorcinol administered by the technique described was also ineffective against these parasites.

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The Microscopic Anatomy of the Skin of Mongrel Dogs

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MOST of the literature concerning skin structure pertains to the human species. Little has been reported on the skin of domestic animals, particularly that of the dog.

German books on the histology of the domestic animals by Ellenberger[4] and Trautmann and Fiebiger[20] gave detailed descriptions of dog skin. Consideration of the sweat glands of dogs has been given by several investigators. Muto[13] and Speed[18] investigated their histology. Hansen *et al.*[7] studied their microscopic structure as a part of a diet program and physiological experiments were conducted by Aoki and Wada.[1] Varicak[21] included the dog in a study of the epidermis of domestic animals. More general texts including references to the dog skin are Miller[11] and Sisson and Grossman.[17]

According to Ellenberger,[4] the skin of the dog showed extensive differences in thickness. Sisson and Grossman[17] pointed out that it was remarkably loose on the dorsal aspect of the neck and trunk where it could be thrown up in large folds. In a small area, the perineum, four types of skin were displayed which were present throughout the body.[12] Miller added that over tuberosities of the ischium, the skin was a thick, tough, resisting type; the scrotal skin was very thin and almost transparent; the anal skin presented a modified type, and there was a general body type of skin over the sides of the perineum. Miller[11] found loosely attached skin on the dorsum of the neck and the thorax. It was thickest in the neck region, thinner over the sternum, and thinnest on the venter of the abdomen. Ham[6] described the skin of man and concluded that skin covering extensor surfaces usually was thicker than that covering flexor surfaces. The skin of the eyelid was thinnest (0.5 mm. or less) and that of the shoulders and back was thickest (up to 5 mm.). According to Piper,[15] bellies of pups had the thinnest skin and the fewest hairs.

According to Varicak,[21] the epidermis of dogs and cats resembled that of man, but the superficial translucent layer was seldom present. The lower stratum lucidum was generally evident.

Trautmann and Fiebiger[20] found a thick panniculus adiposus in the pulplike area on the plantar surface of digits of carnivores. Sisson and Grossman[17] confirmed the findings of Trautmann and Fiebiger.

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HAIR

Ellenberger,[4] Trautmann and Fiebiger,[20] Sisson and Grossman,[17] and St. Clair[19] all agreed that the hair of dogs occurred mainly in groups of threes. Ellenberger[4] gave a detailed description of the structure of hair follicles. He described three-hair groups which consisted of a main hair and two smaller secondary hairs. In the deeper portion, groups of as many as 20 hairs were seen but nearer the surface two to three interposed sebaceous glands divided the hairs so that the three-hair grouping was evident. The division was more evident because of circular connective tissue fibers surrounding the three-hair groups. Trautmann and Fiebiger[20] reported that the follicles of the secondary hairs arose from the follicle of the main hair.

SEBACEOUS GLANDS

Sebaceous glands were found in all breeds of dogs by Ellenberger.[4] They were relatively better developed in dogs having short, rough hair. Sebaceous glands were best developed in the lip, the dorsal side of the rump, and in the sternal region. Trautmann and Fiebiger[20] pointed out that larger oil glands characterized areas having small hairs and that hairless areas were free of sebaceous glands. Sisson and Grossman[17] agreed with Ellenberger and also found well-developed glands in the anus and digital pads.

A group of hair follicles was accompanied by a group of sebaceous glands of approximately the same number.[20]

SWEAT GLANDS

It is believed by many that dogs have no sweat glands; consequently, very little attention has been paid to their structure. The presence of sweat glands in the dog not only in the foot pads but also over the body surface covered with hair was described by Gurlt in 1835.[1] In 1868, Harms found numerous large sweat glands in the dog.[18] According to Speed, Harms stated that the dog can sweat, but the sweat drops are not visible because the hairs are too numerous. This fact probably explains the common misbelief that the dog sweats only on the muzzle and foot pads. Speed[18] gave a very good account of the literature previous to his study.

Ellenberger[4] found sweat glands in all breeds of dogs. Although it was difficult to find them in thick-haired individuals, they were easily seen in the Rat Terrier, the Griffon, the Dachshund, and the Leonberg. Trautmann and Fiebiger[20] observed very small tubular glands which were better devel-

oped in the back, the mouth, the anus, and the foot pads. No tubular glands were found in the end of the nose. Muto [13] called the sweat glands of dogs "bagformed" as opposed to the coiled type in the human species. According to Sisson and Grossman,[17] sweat glands were relatively better developed in the long and fine haired breeds, with the largest glands being found in the foot pads. In the perineum and the paranal pouches, glands were coiled. They added that in the muzzle, glands were absent or few. Saint Clair [19] agreed that sweat glands were few in number and were relatively better developed in long-haired breeds, with the foot pads having the greatest number. Romer [16] stated that sweat glands were generally much reduced in number in carnivores.

Speed [18] examined several breeds—the Airedale, the Cairn Terrier, the Collie, the Greyhound, and the Fox Terrier. He found numerous sweat glands though in some areas glands were absent. There were no glands in the scrotum. The forms varied in different regions but occurred mainly as flexuous tubules which ran nearly parallel with the hair follicles. Those of the digital pad were the typical coiled glands. All glands opened into hair follicles with the exception of those of digital pads.

Aoki and Wada [1] tested the functional activity of sweat glands in the dog. They produced active sweating all over the body with intradermal injections of pilocarpine, acetylcholine, and epinephrine, by local applications of heat, and by direct exposure to excessive heat of the sun. Their results were substantiated by histological findings.

Ellenberger [4] reported funnel-shaped ducts which opened into hair follicles. Trautmann and Fiebiger [20] described sweat glands that had a common duct with sebaceous glands opening into follicles.

Speed [18] confirmed Ellenberger's findings but found separate openings for sweat and sebaceous glands. He further stated that the ducts of glands of the foot pads opened on the surface of the skin.

MATERIALS AND METHODS

Experimental material was obtained from 13 live mongrel dogs which included 8 males and 5 females. The dogs were anesthetized with sodium pentothal. Specimens 1 mm. square, including all layers of the skin and adjacent subcutaneous tissue, were taken from 34 areas over the body. Hansen *et al.*[7] confined their studies to the dorsal surface of the thigh.[8]

The tissues were fixed in Bouin's fluid and were dehydrated and cleared in dioxan. Infiltration was accomplished by three changes of low melting-point paraffin in the oven for half-hour periods. Tissues were embedded in a mixture of one part low melting-point paraffin to two parts "tissuemat." *

Sections of 7 to 10 μ were made and stained. The majority of the sections were cut longitudinally, others were transverse sections.

* Fisher Scientific Co., Pittsburgh, Pa.

Stains used were Harris' hematoxylin and eosin as a routine stain, Wiegert's and Van Gieson's connective tissue stains for elastic and white fibrous tissues, respectively, and Foot's stain for reticulum.[5]

Measurements of epidermis were made at the highest and lowest points in a representative field for each area, and the average of the two figures was recorded in microns. The dermis was measured with average figures being recorded in millimeters.

RESULTS AND DISCUSSION

The skin varied in thickness over the body. Generally, the thickest dermal areas were found beneath the thinnest epidermis. The thickest skin was found in the neck and head regions. The inguinal and axillary regions and the inner lining of the pinna were characterized by the thinnest skin. All of these areas had a thin epidermal covering. The digital pad, which had the thickest epidermis, also had a thick dermis.

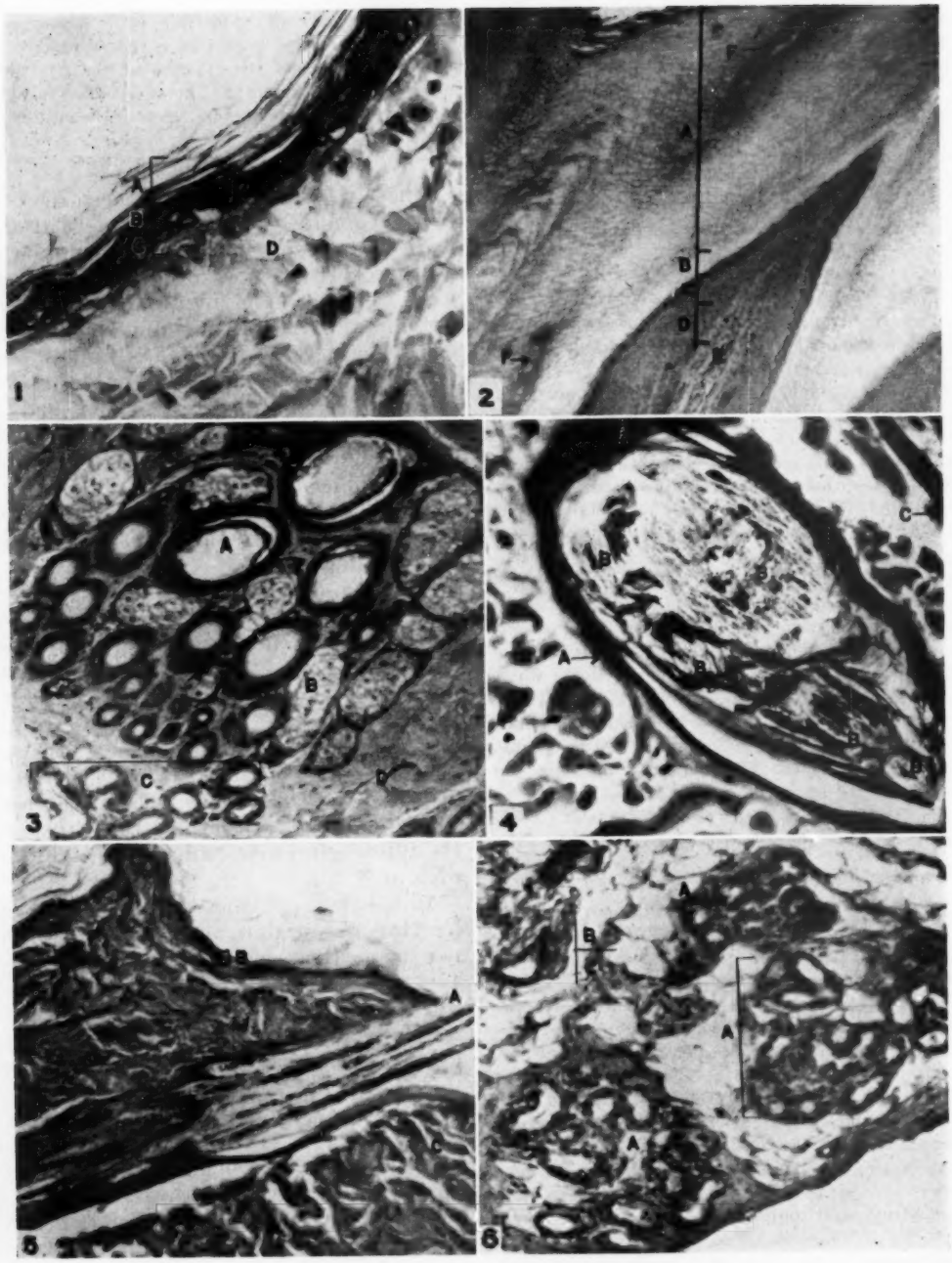
The widest variations were seen in height of the epidermis. Epidermis measuring up to 100 μ was considered to be thin epidermis. That of more than 100 μ was called thick epidermis.

The thinnest epidermis, 26.5 μ in height, was found in the sternal region. The lip, nasal septum, and digital pad presented a thick epidermis. In the lip, it was thickest near the buccal membrane and thinnest at the skin junction, and that of the digital pad reached 1,685.4 μ at the highest point observed. All other areas were characterized by a thin epidermis. The epidermis of the prepuce, the scrotum, the vulva, and the interdigital area ranged in height from 67.5 to 90.0 μ.

In keeping with descriptions of the skin by Ham,[6] thin skin referred to in this paper were areas which had a thin dermis. The term "thin skin" was specifically applied by the author to areas having a dermis which measured less than 1 mm. in height. Areas which had a dermis of more than 1 mm. were designated thick skin.

Due to variations in age and nutritional status of the experimental animals, no measurements were made of the fat layer. However, the author observed a general increase in thickness of the fat layer in females. No other sex differences were distinguished.

Epidermis.—Four distinct epidermal layers were found, stratum germinativum, stratum granulosum, stratum lucidum, and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stratum corneum. The layers were similar to descriptions of those of the human species.

The stratum germinativum, which consisted of the basal layer and the stratum spinosum, was present in all areas. It varied in thickness from the two cell rows of the mandibular, maxillary, and temporal regions, the dorsum of the head, the ear, and the back to as many as 35 rows of cells in the nasal septum. The cells of the basal layer were columnar in shape with some cells being lower in height than others. In the thicker areas, the deeper cells of the stratum spinosum were domeshaped. Those more superficially located were polyhedral in shape. Tonofibrils characteristic of this region were evident.

The stratum granulosum was generally present. It consisted of a single row of cells in the venter of the neck, the abdominal, sternal, axillary, and shoulder regions, the flank, the tail, and the back (fig. 1). It was absent in the mandibular and temporal regions, dorsum of the head, and outer covering of the ear. The stratum granulosum in the digital pad, which was the thickest observed, consisted of 15 rows of cells. The cells contained keratohyalin granules in their cytoplasm. In the thickest epidermis, shapes ranged from polyhedral in the deepest row to the diamond shaped in the most superficial row. Diamond-shaped cells characterized areas having but a single row of cells.

The stratum lucidum was best developed in the digital pad (fig. 2). It was absent in the head, neck, shoulder, axillary, sternal, and abdominal regions, the back, the tail, and the prepuce.

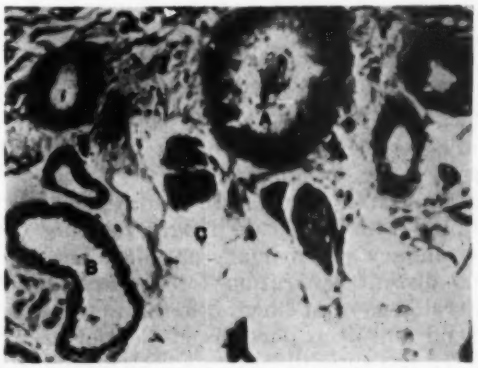

Fig. 7 — Cross section from temporal region showing tubular (noncoiled) sweat glands in the dermis. (A) Hair follicle; (B) sweat gland; (C) fat. Hematoxylin-eosin stain. x 90.

Observations showed a stratum corneum in all areas studied. It varied from 5.3 μ in the lip to 1.5 mm. (1,526.4 μ) in the digital pad. The stratum corneum of the nose was 106 μ in width. That of the ear, mandibular region, prepuce, scrotum, and anus was approximately 21.2 μ wide. The stratum corneum of all other areas averaged 60 μ in width. In the digital pad, cell boundaries were seen. Faint appearing nuclei were in the layers nearest the stratum lucidum (fig. 2). The stratum corneum of the lip was reduced to a pale staining line.

White or light-colored skin was characterized by a few melanin granules in the cytoplasm of the basal layer of the stratum germinativum. The amount of pigment in-

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Fig. 1—Section from back showing thin epidermis. (A) Stratum corneum; (B) stratum granulosum; (C) stratum germinativum; (D) dermis. Hematoxylin-eosin stain. x 300.

Fig. 2—Epidermis of the digital pad showing the thick stratum corneum and stratum lucidum. (A) Stratum corneum; (B) stratum lucidum; (C) stratum granulosum; (D) stratum germinativum; (E) dermis; (F) duct of sweat gland. Hematoxylin-eosin stain. x 75.

Fig. 3—Cross section of dorsum of head showing a group of hair follicles, sebaceous glands, and sweat glands. (A) Hair follicle; (B) sebaceous gland; (C) sweat glands; (D) dermis. Hematoxylin-eosin stain. x 135.

Fig. 4—Cross section of back showing a group of six hairs in one hair follicle. (A) Hair follicle; (B) hair; (C) M. arrector pili. Hematoxylin-eosin stain. x 250.

Fig. 5—Longitudinal section of tail showing five hairs emerging through a common opening in the skin. (A) Hairs; (B) epidermis; (C) dermis. Hematoxylin-eosin stain. x 115.

Fig. 6—Section of digital pad showing groups of coiled sweat glands in the dermis and the tela subcutanea. (A) Sweat glands; (B) dermis; (C) tela subcutanea. Hematoxylin-eosin stain. x 90.

creased with darkness of the skin. Black skin had melanin in all layers of the epidermis.

The author was able to impregnate the basement membrane with silver. This was in agreement with Dempsey[3] and Odland[14] that it was a reticulum.

Dermis.—The surface of the dermis was raised into numerous ridges forming both secondary and primary papillae. There was no distant separation into layers but the arrangement of fibers differed in the superficial and deep portions of the dermis.

In the superficial or papillary zone, elastic fibers were generally finer and were woven into a network, though single fibers were found. Bundles of collagenous fibers varied in thickness and arrangement. Small, deeply staining fiber bundles, located nearer the epithelial covering, were parallel to the surface. A few fibers extended in every direction.

The fibers of the deep or reticular layer of the dermis were coarser than those of the papillary layer. Elastic fibers were more numerous and fewer networks were seen. The collagenous bundles were wider and fibers stained paler than in the papillary zone.

Fine reticular fibers were seen throughout the dermis. Numerous fibers appeared just beneath the basement membrane and around hair follicles.

In areas where the skin was most pliable, such as the axillary and flank regions, and the dorsum of the neck, collagenous bundles were smaller and more loosely arranged. Elastic fibers in the same areas were relatively more numerous in the superficial layer than in the deep layer. Wider, more closely packed collagenous bundles and fewer elastic fibers characterized the superficial layer in areas where the skin was least movable, such as the tail, the ear, and the digital pad. The elastic fibers of the deep layer of the inguinal region were larger than those in the superficial layer.

Although collagenous fibers passed in every direction, large, pale-staining bundles which were roughly parallel to the surface occurred in areas of thick skin such

Fig. 8—Skin from back showing coiled sweat gland in tela subcutanea and dermis. (A) Epidermis; (B) dermis; (C) tela subcutanea; (1) hair follicle; (2) sebaceous glands; (3) opening of sebaceous glands; (4) sweat gland; (5) duct of sweat gland; (6) M. arrector pili. Hematoxylin-eosin stain. x 90.

as the head regions, digital pad, lip, nasal septum, and dorsal surfaces of the body. Bundles, which were smaller and dark staining, characterized thin areas for the most part.

In many sections, pigment laden cells were seen in portions of the dermis near the basement membrane. No correlation was made between amounts of pigment in the dermis and pigment content of epidermal cells.

Numerous large nerve trunks were observed, especially in the deep layer of the dermis and in the subcutaneous tissue. Smaller nerve trunks were seen in both dermal layers. No specialized nerve endings appeared in the sections examined by the author. Such endings probably are located in the dermis, but stains other than those employed in this research as well as other methods of study should be used. Serial sections probably would be of value in such a study.

Hair.—The author's findings agreed with those of Ellenberger [4] as to grouping of hair follicles. Ellenberger found up to 20 hairs in a single group located deeply in the dermis. Higher up, they were separated into three hair groups by two to three sebaceous glands and circular connective tissue fibers. The author found more sebaceous glands separating the follicle groups (fig. 3) than did Ellenberger. This could have been due to the plane of sectioning or Ellenberger may have been referring to groups of sebaceous glands. Figure 4 shows one main hair and five accessory hairs in a single group. Although the arrangement of hair into groups was the general picture (fig. 3, 4, and 5), single hairs were found in the lip. Occasionally, single hairs were also found in areas which showed a definite grouping as the rule (fig. 8). The digital pad, nasal septum, and portions of the lip were free of hair.

Sebaceous Glands.—Sebaceous glands were always associated with hair follicles, consequently no glands were seen in hairless areas. Observations made on the sebaceous glands indicated that they were generally larger than those of the human species (fig. 3). Glands of approximately the same size as those in man, as well as smaller ones, were seen. The general structure compared with that of glands described by Bailey,[2] Lambert,[9] Maximow and Bloom,[10] and Ham.[6] Sebaceous glands opened into hair follicles (fig. 8).

Sweat Glands.—Every area studied presented sweat glands with the exception of the nasal septum. In the lip, glands were evident only in hairy portions. Speed [18] described the scrotum as being free of sweat glands. The author found sweat glands in each section of the scrotum from 8 dogs.

Sweat glands in the dog were of two main types. One was the typical coiled arrangement described by previous authors (fig. 3, 6, and 8); the other was a simple tortuous tube or noncoiled type with a large lumen (fig. 7). The structure of the tubular type compared with that of the bag form described by Muto.[13] The lip and digital pad had the coiled type only. Coiled and noncoiled glands were found in all other areas in which sweat glands were observed. The glands of the digital pad opened onto the surface of the skin (fig. 2). All other glands opened into hair follicles.

SUMMARY AND CONCLUSIONS

Specimens of dog skin from 34 body areas of mongrel dogs were studied. Experimental animals included 8 males and 5 females. Other than a thicker fat layer in females, no sex differences were observed.

Thick skin, generally, had a thin epidermis and a thick dermis (1 mm. or more). The skin was thickest in the head and neck regions, although the dorsal and lateral surfaces of the body also were characterized by thick skin. The thinnest skin was found in the lining of the pinna and the inguinal and axillary regions. There seemed to be no consistent pattern as to thickness or thinness of epidermis over thin dermal areas. The thin dermis of the lining of the pinna, and the axillary and inguinal regions were covered by thin epidermis. The head, neck, shoulder, axillary, sternal and abdominal regions, the back, tail, and prepuce consisted of thick dermis underlying thin epidermis. The lip and the nasal septum had a thick epidermal covering over thin dermis. Thick epidermis covered the thick dermis of digital pads. Skin covering ventral and medial surfaces was about 1 mm. thick, some areas being more and some less, but in all of these areas the epithelium was thin.

The layers of the epidermis compared

with general descriptions by other authors. All four layers were found in thick epidermis. The stratum germinativum was present in all areas though it varied from two cell rows in the head and neck regions to 35 rows in the nasal septum. The number of cell rows increased with thickness in epidermis. With the exception of its absence in the head regions, the stratum granulosum was present in all areas. It consisted of a single row of diamond-shaped cells on ventral surfaces of the body, the tail and the shoulder. At its thickest point, the digital pad had 15 rows of cells in the stratum granulosum. The thin epidermis of the head, neck, shoulder, axillary, sternal and abdominal regions, the back, the tail, and the prepuce had no stratum lucidum. It was thickest in the digital pad. The stratum corneum, which was thinnest in the lip, attained its greatest development in the digital pad. It was present in all sections studied.

The amount of pigment varied with skin color. In white or light-colored areas, pigment was found mainly in the stratum germinativum but in black skin it was found in all layers of the epidermis. It was also observed in the papillary layer of the dermis.

The dermis showed no distinct separation into layers. Fine elastic and collagenous fibers characterized the superficial dermal layer in all areas. Coarser fibers were found in the deep layer. Throughout the dermis were fine reticular fibers. They were more numerous around hair follicles and the basement membrane. In thick-skinned areas, bundles of collagenous fibers were generally larger and stained lighter. In thinner areas, fiber bundles were smaller and took a deeper stain. Fibers extended in all directions but in thicker areas were more parallel to the surface. Bundles were loosely packed in the flank and inguinal regions as compared with densely packed bundles of less movable areas.

Hair was absent in the digital pad, nasal septum, and portions of the lip. All other areas had hair. Hair follicles occurred mainly in groups of threes. Deep in the dermis, groups were massed together giving the appearance of as many as 20 hairs in a single group. More superficially sebaceous glands and circular connective tissue fibers separated hairs into smaller groups.

Sebaceous glands compared with descriptions of those of the human species. All hairy portions of the body presented sebaceous glands. They were generally larger than in the human species but glands of several sizes were found.

Numerous sweat glands were found in all areas studied with the exception of the nasal septum and nonhairy parts of the lip. The glands were of two types—coiled and noncoiled. Both coiled and noncoiled glands were distributed over the entire body with the exception that noncoiled glands were not found in the digital pad and hairy parts of the lips.

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The Effect of Certain Chemicals on the Eggs and Larvae of the Canine Hookworm (*Ancylostoma Caninum*)

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THE CONTROL of hookworm larvae in soil has long been recognized as a logical approach to the control of hookworm disease. Much work has been done on the testing of chemicals as larvicides for hookworm and other nematodes and numerous papers have reported the results.

LITERATURE CITED

For extensive reviews of the literature, reference is made to the papers of Parnell,[1,2] Levine,[3,4,5] Ackert and Ligenzowski,[6] and Hoerlein.[9] Underwood[6] reported the usefulness of NaCl in killing hookworm larvae in soil and this method is still perhaps the most practical one where soil productivity is not involved. Stoll[7] found that fairly heavy concentrations of lime in night soil killed human hookworm larvae. Ackert and Ligenzowski[8] found 10 per cent acetic acid to be effective in cat fecal cultures and Hoerlein[9] found methyl bromide and acid sodium borate to be effective on dog hookworm larvae. In 1949, Levine[3] reported on the use of a large number of soil fumigants in killing horse strongyle larvae. He found mercuric chloride, sodium arsenite, nicotine sulfate, potassium iodide, sodium iodide, and iodoform to be the most effective of all of those chemicals tried. Later, Levine[4,5] reported on the effects of other iodine compounds as well as organometallic and other heavy metal compounds on horse strongyles and suggested that some of these compounds may be useful in destroying hookworm larvae.

The work reported here involves the testing of some chemicals which have previously been used as nematocides, others which are known best, perhaps, as acaricides or molluscacides, and some known best as insecticides, as well as for their use in fish management. The nematocides are ethylene dibromide, dowicide G, premerge, and dowfume N. The acaricides are DN I and DN III. The insecticides are rotenone, lexone, and chlordane. The latter have recently been used in various aspects of fish management, rotenone to kill fish, and lexone and chlordane to control invertebrates in fish ponds.

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MATERIALS AND METHODS

A steady supply of eggs was obtained from experimentally infected dogs. Fresh stools were mixed with water to a smooth, liquid consistency. From this mixture, equal portions were placed in 4-in. finger bowls with approximately 100 cc. of quartz sand with a grain size of about 0.5 mm. Two small Stender dishes of tap water were added to each culture. Each culture was mixed and covered with a glass plate which, unless otherwise mentioned, was left on for the duration of the experiment. In each experiment, half of the cultures were treated immediately after preparation and half were used as controls.

The methods of treating the cultures were spraying, dusting, mixing, and pipetting. If the cultures were to be sprayed, the required amount of experimental solution was placed in a nasal atomizer, and an attempt was made to see that all areas of the culture received an equal amount of spray.

A special apparatus was constructed for the dusting which involved blowing the powdered chemical into a cloud under a large beaker inverted over the culture dish. This produced an even distribution of dust over the surface of the culture. Mixing involved stirring the chemical evenly through the sand and feces mixture; in pipetting, the chemical was merely released from a pipette onto the surface of the culture.

Larvae were concentrated by a modified Baerman technique. The cultures were dumped onto squares of cheesecloth, four layers thick; the corners of these were picked up, and the whole put into brass wire inserts placed in glass funnels filled with water. The funnels were allowed to stand for about six hours. The water from the bottom of the funnel neck was then drained into a small Stender dish and the larvae counted under a dissecting microscope at a magnification of 30. Larvae not showing any sign of movement were considered to be dead.

In certain DN I experiments, the covering plate of glass was left off after treatment. These cultures were sprayed with water at intervals to prevent drying out. Another variation with DN I was to treat the cultures and then, instead of covering them with glass plates, they were placed under large bell jars. This was done to prevent the moisture that normally collects on the glass plates from attracting the larvae.

In another experiment, twenty-four cultures were dumped onto a large glass plate and this molded into a "mud pie" at least 10 cm. thick. Twelve cubic centimeters of ethylene dibromide

were sprayed on this cake. The cake was then covered with a plastic sheet and the sides of this held down so that it was reasonably air tight. In one experiment, triton was added to the ethylene dibromide in an attempt to emulsify it but with only partial success.

In the DN I, DN III, rotenone, and benzene hexachloride experiments, the chemical was applied to the cultures when they were established. In all others, the chemical was applied after the larvae appeared.

RESULTS

A total of 124 experiments involving 2,350 separate cultures was employed in obtaining the results reported here. Since so much space would be required to report on each experiment, only the optimum results and, in certain cases, a comparison of results obtained by different methods of application are given (table 1). Tables giving complete results of all 124 experiments are permanently deposited in the Biology Library at the University of Oklahoma.

1) *Ethylene Dibromide.*—Twenty-two experiments involving 380 cultures were performed with ethylene dibromide. It was used in doses ranging from 0.125 cc. to 0.5 cc. per culture. Doses were both sprayed and pipetted onto the cultures and after treatment different cultures were kept covered, left uncovered, and some made into sand cakes under a plastic canopy. The optimum results are illustrated by the sample experiments given in table 1. A minimum of 0.5 cc. sprayed or pipetted onto the culture will give significant kills within twenty-eight to seventy-two hours.

2) *Dowfume N, 1, 3 Dichloropropene.*—Ten experiments involving 200 cultures gave significant kills when 0.5 cc. was pipetted onto the culture. The same amount sprayed onto the culture did not give satisfactory results. In this case, the cultures were all left covered because of the volatility and toxicity of the chemical. Total kills were obtained within twenty hours.

3) *Premerge, Alkanolamine Salts (of the Ethanol and Isopropyl Series) of Dinitro-o-sec-butylphenol, 54 Per Cent.*—In five experiments involving 126 cultures with doses as high as 0.75 cc. per culture, no significant kills were obtained. In certain cases, there seemed to be a reduction in numbers but in others there were more larvae in the experimental cultures than in the controls.

4) *Dowicide G, Sodiumpentachlorophenate, 75 Per Cent, Sodium Salts of Other Chlorophenols, 13 Per Cent.*—One-half cubic centimeter of a 20 per cent solution sprayed on each culture gave significant kills after fifty-four hours, or 1 cc. of a 30 per cent solution gave significant kills within twenty-four hours after application. These results are based on ten experiments involving 192 cultures.

5) *DN I, Dinitro-o-cyclohexylphenol, 40 Per Cent.*—In 21 experiments involving 307 cultures, significant kills were obtained when more than 300 mg. was mixed with the soil in each culture although one week of exposure was necessary to reduce the number of experimental larvae significantly below that of the controls. Smaller doses dusted or mixed with the culture did not produce satisfactory kills.

6) *DN III, Dicyclohexylamine Salt of Dinitro-o-cyclohexylphenol, 20 Per Cent.*—In six experiments involving 78 cultures, results were so poor that the experiments were discontinued. The best results were obtained when more than 464 mg. was mixed with each culture. After seven days, there was an apparent reduction in the

TABLE 1—Optimum Results from Chemicals Tested

Chemical	No. cultures	Dosage per culture	Method of treatment	Time treated	Results Live larvae count	
					Experimentals	Controls
Ethylene dibromide	16	0.5 cc.	sprayed	72 hr.	9	3,045
	20	0.5 cc.	pipetted	28 hr.	186	32,000
Dowfume N	14	0.25 cc.	pipetted	19.5 hr.	28	15,200
Premerge	31	0.75 cc.	sprayed	18 hr.	506	3,960
	14	0.50 cc.	pipetted	19.5 hr.	10,000	14,100
Dowicide G	18	0.5 cc. (20 %)	sprayed	54 hr.	57	3,167
DN I	22	303.87 mg.	mixed	8 days	151	13,850
DN III	14	463.98 mg.	mixed	7 days	2,050	9,850
Crude rotenone (5% +)	48	126.61 mg.	mixed	7 to 8 days	773	17,500
Pure rotenone	40	82.2 mg.	mixed	8 to 9 days	21,500	33,300
Benzene hexachloride	42	100.38 mg.	mixed	6 to 7 days	1,193	7,325

number of larvae in experimental cultures but the differences were not significant.

7) *Derris Root, Crude Rotenone Containing at Least 5 Per Cent Pure Rotenone.*—In cultures treated with 126 mg., significant kills of hookworm larvae were obtained within six to seven days after treatment. Results were not consistent in all experiments. In one involving 28 cultures, 84.42 mg. per culture gave significant kills in the same length of time. On the other hand, in other experiments 126 mg. did not give significant kills. All of the crude rotenone used was from the same supply and presumed to be of the same percentage but the results indicate that this may not have been the case.

8) *Pure Rotenone ($C_{23}H_{22}O_6$).*—The pure rotenone gave poor results as compared to the crude rotenone. Only the one experiment shown in the table produced a significant kill.

9) *Chlordane ($C_{10}H_6Cl_8$).*—In three experiments involving 80 cultures with this chemical, no effect on the larvae was demonstrated and the experiments on it were discontinued.

10) *Lexone, Benzene Hexachloride (Gamma Isomer, 10%; Other Isomers, 53%).*—Significant kills were obtained when cultures were treated with 100.38 mg. of the powdered chemical. When the chemical was dissolved in water and poured on the cultures or when it was dusted on them, the results were poor.

DISCUSSION

In performing experiments of this type, one soon is aware of the fact that the method by which the chemical is applied may determine its effectiveness. For example, in the use of ethylene dibromide, dowfume N, and dowicide G, the effectiveness depends on retaining the highly volatile chemical in the soil long enough to kill the eggs or larvae. There is little question that they are highly effective if the soil is covered by a canopy after treatment. Injection into soil as is done with many volatile nematocides would undoubtedly be effective although this method was not used in any of our experiments.

The principal difficulty with ethylene dibromide, dowfume N, and dowicide G in controlling hookworm is that they can not be used easily by untrained people, and a certain amount of usually unavailable equipment is necessary for their use. It is questionable if they should be advised in preference to sodium chloride, the effectiveness of which is well proved, provided the sodium chloride can be applied without danger of other damage. Benzene hexachloride could prove useful in treating manure piles, kennel runs, etcetera, where sodium chloride is not advised.

Although the DN I compound proved to be effective against hookworm, the amounts necessary for effective kills were extremely high. Its effects are definitely increased by mixing with the soil, and there is inconclusive evidence that, when this is done, the hatching of the eggs is definitely inhibited. The effects, therefore, may be on the eggs and not the larvae since the latter will live for several days in a saturated solution.

The interesting observation to be made on rotenone is that since the crude form gave definitely better results than the purified form, rotenone may not be the active principle; contaminants in the crude rotenone may be the effective agents.

SUMMARY

One hundred twenty-four experiments involving 2,350 cultures of canine hookworm *(Ancylostoma caninum)* larvae were conducted in testing the effects of nine chemicals on the larvae.

Cultures were prepared by mixing dog feces with 100 cc. of quartz sand in a 10-cm. finger bowl. Larvae were separated from the culture by a modified Baerman technique.

Ethylene dibromide, dowfume N, and dowicide G gave effective kills when sprayed on the culture, provided the cultures were covered.

DN I, benzene hexachloride, and crude rotenone were found to be effective only when very large amounts were used. DN III, premerge, and chlorodane were not effective, and pure rotenone produced poor results.

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Glomerular Lipoidosis in the Dog

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A PUBLISHED comment to the editor of *Science* [1] stressed the normal or physiological occurrence of lipid deposits in the epithelial cells of the proximal convoluted tubules of the canine kidney.

It is of historical interest that this observation was made almost a century ago by Vulpian [2] in 1862. Silver's report was prompted by that of Dallemagne, Gerebtzoff, and Philippot [3] who claimed that these intracellular fat deposits occurred in this location as the result of the administration of hexachlorocyclohexane to dogs. It is generally believed that fatty changes observed in the canine kidney must be seen in greater quantity or in locations other than the epithelial cells of the proximal convoluted tubules (distal portion) before such change is considered pathological.

In the course of a study utilizing dogs as the test animal, we encountered an unusual glomerular lesion characterized by large deposits of lipid within the cytoplasm of the endothelial cells of the glomerular capillaries. The animal was a male mongrel approximately 3 years of age who was maintained on the usual laboratory diet of meat scraps and chow checkers. He was chosen for the experiment of total hepatectomy because of his good physical condition, but he expired unexpectedly during the administration of ether anesthesia before any operative procedure was undertaken. He had not been subjected to any other drugs or previous experimentation. A catheterized urine sample taken prior to death was negative for albumen and reducing substances and demonstrated a normal sediment. The organs were taken for microscopic study as controls.

Grossly, the kidneys revealed thin capsules which stripped with ease revealing slightly swollen, reddish brown cortices. Their cut surfaces revealed well-defined cortical and medullary substance which also was reddish brown except for slightly yellowish tan cortical columns.

Microscopically, approximately one half of the glomeruli showed the endothelial cells of the capillary tufts to be markedly swollen with vacuolated cytoplasms and centrally placed nuclei (fig. 1). An oil red O stain for lipid performed on frozen sections of formalin-fixed (10 %) tissue demonstrated abundant deposits of lipid within the cytoplasm of these swollen endothelial cells as well as a normal quantity of lipid in the epithelial cells of the distal portion of the proximal convoluted tubules (fig. 2). This material was not evident in sections similarly stained after paraffin embedding. It was not birefringent. A rare glomerular epithelial cell also revealed a similar deposit as well as hyaline droplets within its cytoplasm. These endothelial and epithelial changes were frequently observed focally within an otherwise normal glomerulus as well as replacing the entire glomerular ap-

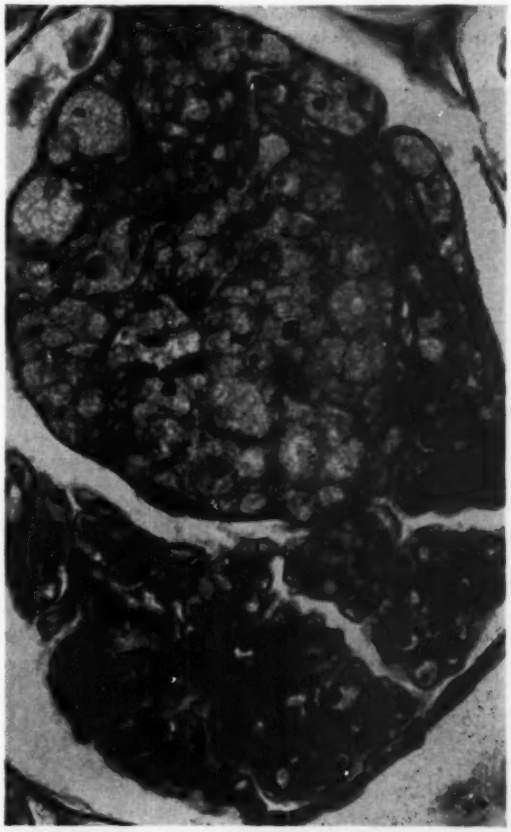

Fig. 1—Photomicrograph of involved glomerulus revealing focus in which endothelial cell cytoplasm is swollen and vacuolated. x 450.

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paratus. The capsular spaces were free of change except on occasion when these spaces were obliterated by the swollen endothelial cells. The basement membranes of the glomerular tufts appeared to be of normal thickness as demonstrated by the periodic acid-Schiff stain. The tubular epithelium of the proximal convoluted tubules revealed normal-appearing brush borders and normal alkaline phosphatase activity. There were no other vascular or stromal changes noted in the many sections studied. Sections of other organs failed to reveal pathological change.

COMMENT

The occurrence of spontaneous renal disease in dogs is not infrequent. Bloom,[4] in an exhaustive study, cited the occurrence of clinical renal disease in 274 of 4,123 dogs of all ages and breeds. In another group of 200 that died of unsuspected kidney disease or other causes, 108 demonstrated renal lesions. However, he stated that the occurrence of glomerular lesions is most uncommon, the vast majority of involved kidneys revealing an interstitial nephritis. No lesion similar to that reported here was mentioned and a search of the literature failed to disclose such a case. In a personal communication, Bloom [5] stated that he has occasionally observed this glomerular lesion in otherwise normal dogs as well as those having other renal lesions. He also has been unable to find any mention or description of this unusual glomerular change in the literature.

It is of interest that a similar lesion is illustrated in Allen's monograph, "The Kidney,"[6] (p. 155). However, the photomicrographs were obtained from sections of the kidney of an infant who died with the clinical manifestations of the nephrotic syndrome.

The pathogenesis of this glomerular change is not apparent although it seems logical to assume that it is spontaneous and perhaps is of a degenerative nature. Instances of fat embolism to the kidney observed by us reveal an eccentric and frequently obliterated nucleus in the endothelial cell. Often, the glomeruli are devoid of recognizable endothelial cells. In addition, there was no reason for such a phenomenon to have occurred in this animal. The possibility of the ether inhalation acting as the exciting factor may be dismissed, since we have not observed any similar changes in other animals although ether anesthesia is routinely employed. Further, the age of the lesion, morphologically, exonerates the anesthetic as being of etiological significance.

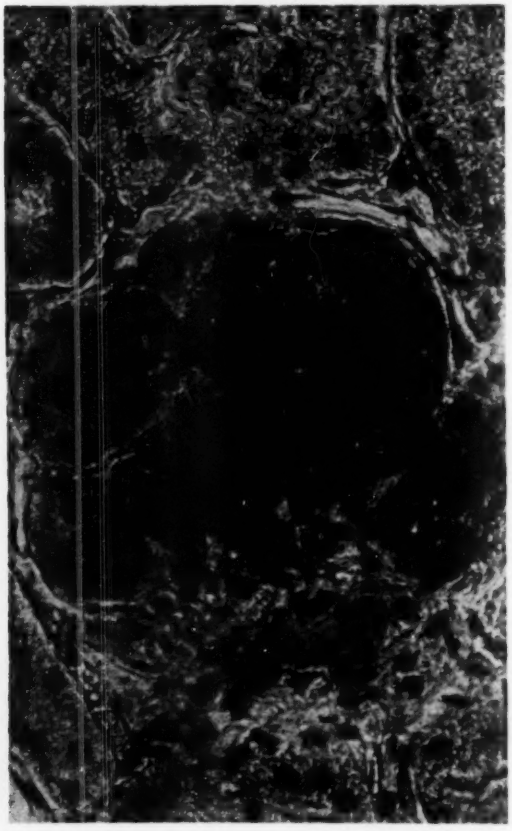

Fig. 2—Oil red O stain demonstrating lipid within glomerulus. x 185.

CONCLUSION

We must conclude that the occurrence of lipid within the canine glomerulus may be spontaneous in an otherwise normal animal. One must be as cautious in ascribing experimental significance to such a lesion as to fatty tubular change in the dog.

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Aërosol Transmission of Newcastle Disease in Chickens

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NEWCASTLE disease virus (NDV) is generally believed to be transmitted through the air because of the rapid spread of the disease under farm as well as experimental environments. In 1947, Idani and Seetharaman [1] showed that susceptible birds would get the disease when held for three hours or more in pens separated by a distance of not more than 2 ft. from enclosures containing fowl infected with Newcastle disease (ND). The following year, Delay and co-workers [2] reported the infectivity of the air of poultry houses harboring chickens infected with Newcastle disease. The virus was recovered by passing a measured volume of air through normal allantoic fluids and then inoculating the fluid into embryonating eggs. Normal chickens which inhaled the air of the contaminated poultry house developed typical signs of ND a few days later, and the blood of 3 of the 4 chickens possessed hemagglutination inhibition antibodies on the eighth day. On challenge of the 4 birds with active virus on the fifteenth day, all were refractory to infection. The virus was also demonstrated in dust collected with a vacuum cleaner from the air of the poultry house.

The present report is a preliminary attempt to ascertain the concentration of virus in such expired air in the different stages of the disease. The methods used are discussed.

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Published with the approval of the director of the Wisconsin Experimental Station.

MATERIALS AND METHODS

Strain GB of NDV was used in these trials. The strain was isolated from an outbreak of highly fatal Newcastle disease among chickens in Texas.[3]

Chickens of the New Hampshire breed, 6 to 8 weeks old, both male and female, were employed. These birds had never shown clinical signs of ND and their blood serums were negative to tests for hemagglutination inhibition activity.

To produce uniform clouds of the virus particles for exposure of the chickens to NDV, a modification of the method previously described was utilized.[4] A Cenco vacuum-pressure pump, instead of a manually operated rubber bulb, provided a constant air pressure. Using 3 lb. of air pressure, approximately 0.03 ml. of virus suspension of infected allantoic fluids in tryptone broth was atomized into the air during each minute of operations (fig. 1).

The air in the vicinity of infected chickens was sampled in three ways: In the first, an air-sampling device was connected to the vacuum line of

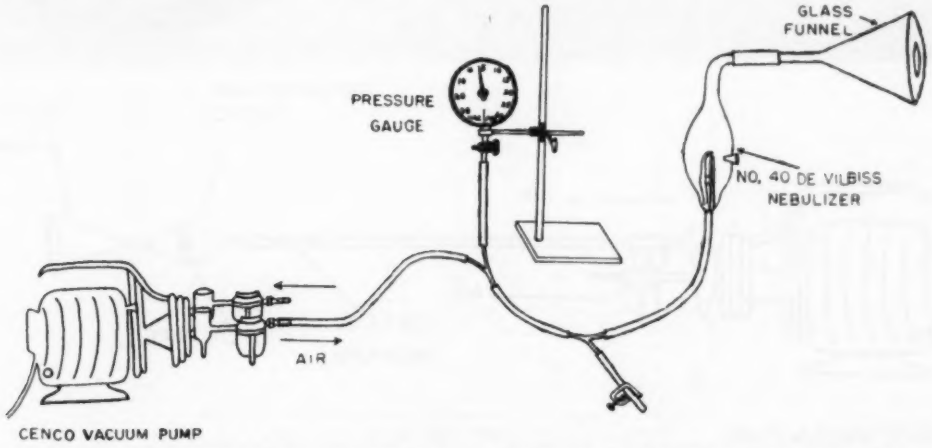


Fig. 1—Apparatus employed for aërosols of Newcastle disease virus in the air.

the pressure pump. The device consisted of a metal membrane holder and a critical aperture* which permitted 4.97 liters of air to pass through the filter per minute of operation of the pump. The virus particles were collected upon the membrane of the special filter paper.** A wide-mouthed funnel with a short neck was attached to the air-sampling device to facilitate collection. In the second, the same apparatus was used but the filter paper membrane was replaced with a filter of gelfoam.®† In the third method, a liquid impinger was employed to sample a measured volume of air by bubbling it through 10 ml. of normal allantoic fluid. The apparatus for sampling a measured volume of air from the infected birds is shown in figures 2a and 2b.

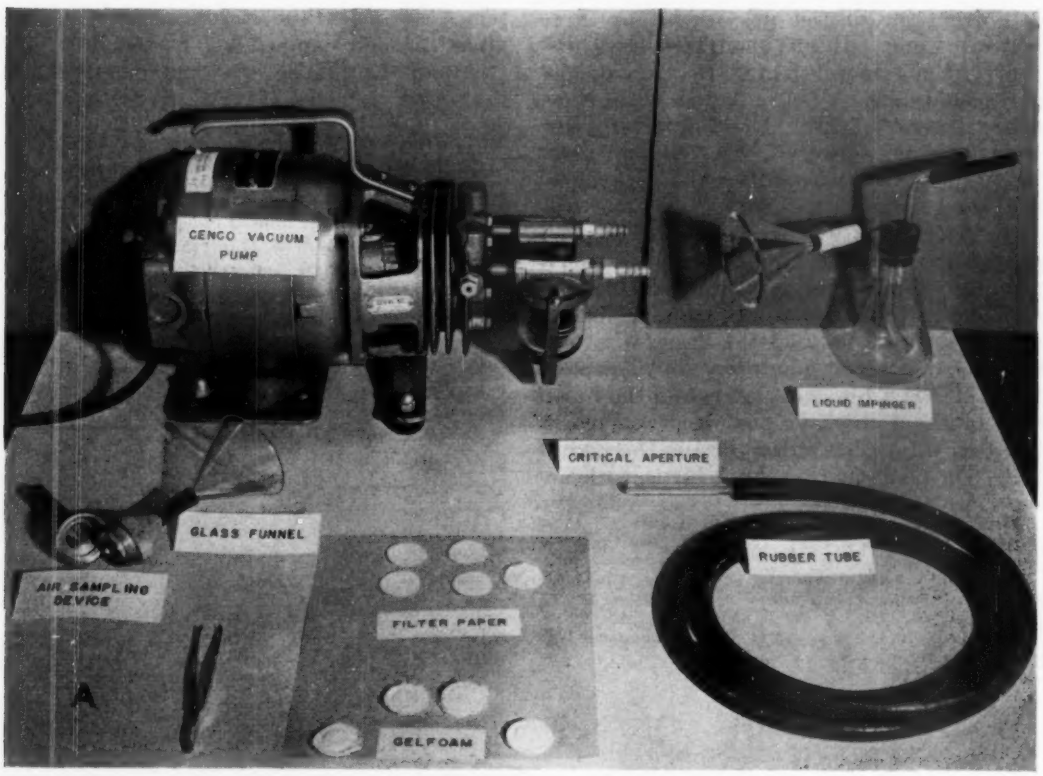
* Prepared by Camp Detrick Biological Laboratory, Maryland.

** Filter papers of 0.1 μ porosity, 1 in. in diameter, were supplied by Dr. W. R. Hinshaw, Frederick, Md.

EXPERIMENTAL

Two trials were made simultaneously in the main experimental room. For each trial, 16 birds

† Prepared by the Upjohn Company, Kalamazoo, Mich.



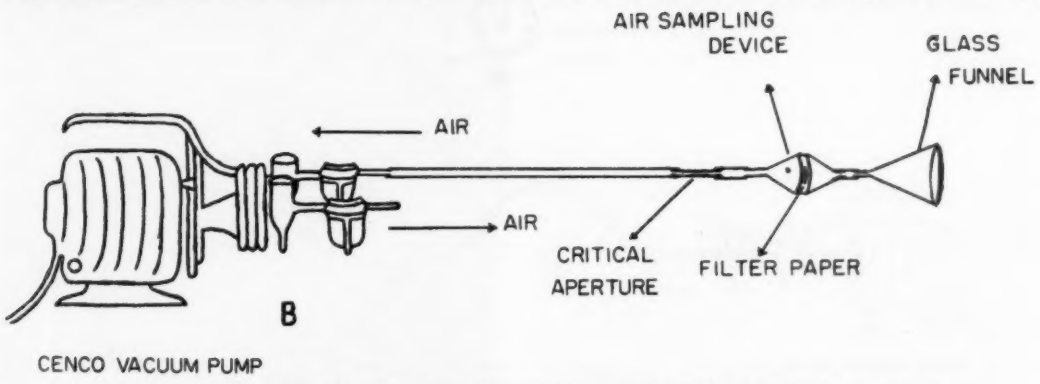


Fig. 2—Apparatus employed for isolation of Newcastle disease virus from the air.

were separated into four equal groups, A, B, C, and D. Each group was placed in a separate cage (1½ by 1½ by 2 ft.). The cages were placed on a support 1½ ft. from the floor and spaced at distances of 1 ft. from each other in such a manner that contact through feed or drinking water was avoided and possible indirect contact was reduced to a minimum. After exposure of groups A and D to NDV by aërosols in another room, the holding cages were placed on the support in the main experimental room. Cages containing the unexposed control birds, groups B and C, were placed in the center of the cage support, so that they would be between groups A and D. Critical observations were made daily for fifteen days following exposure.

Samples of air were collected from the cages every day for six days after the birds were exposed to the virus. Approximately 25 liters of air were sampled through each of the filtering devices. The filtering membranes or fluids were stored at —20 C. In testing for virus, each filter paper and gelfoam disk was macerated in 10 ml. of tryptone broth, the suspension centrifuged at 1,500 r.p.m. for ten minutes and treated with antibiotics. The antibiotic solution, consisting of 5,000 mg. of streptomycin and 5,000 units of penicillin per milliliter, was added to the inoculum to suppress bacterial growth. The normal allantoic fluid through which the sampled air was bubbled was treated similarly with antibiotics. The supernatant fluid was approximately a 10^{-1} dilution and 0.2 ml. of it was inoculated intra-allantoically into each of 4, 10-day-old embryonating eggs. Allantoic fluids were harvested from the eggs that died within five days and each was tested for the presence of virus by the hemagglutination test.

TABLE 1—Development of Newcastle Disease in Chickens After Exposure to Artificial Aërosols

Trial group		Bird (No.)	Days after initiation of experiment 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
		9549	....	....	S	S	D										
A	1	9521	....	....	....	S	S	M	D								
		9519	....	....	S	S	S	D									
		9538	....	....	....	W	S	M	D								
		9625	....	....	....	S	S	M	D								
	2	9571	....	....	W	S	S	M	D								
		9478	....	....	W	S	S	D									
		9589	....	....	....	S	S	M	D								
		9565	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
D	1	9604	....	....	....	W	S	S	D								
		9542	....	....	W	S	S	D									
		9610	....	....	W	S	S	D									
		9607	....	....	....	W	S	S	D								
	2	9545	....	....	....	S	S	D									
		9594	....	....	W	W	S	M	D								
		9513	....	....	S	S	D										

Average time until signs appeared—4.1 days.

Development of Newcastle Disease in Chickens After Exposure to Natural Aërosols (Source of Infection Was Air from Cages of Chickens Developing First Signs on Day 3 and Dying by Day 7)

Trial group		Bird (No.)	Days after initiation of experiment 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
		9473	....	....	....	....	....	....	S	S	D						
B	1	9479	....	....	....	....	....	....	S	S	S	D					
		9485	....	....	....	....	....	W	S	S	D						
		9488	....	....	....	....	....	....	....	S	S	M	D				
		9402	....	....	....	....	....	....	W	S	S	S	D				
	2	9493	....	....	....	....	....	....	W	S	S	S	D				
		9497	....	....	....	....	....	....	....	....	....	S	S	D			
		9499	....	....	....	....	....	....	....	....	....	....	S	S	S	D	
		9469	....	....	....	....	....	....	....	....	....	....	S	S	D		
C	1	9471	....	....	....	....	....	....	....	....	....	W	S	S	S	M	D
		9470	....	....	....	....	....	W	S	D							
		9482	....	....	....	....	....	....	....	S	S	S	(destroyed)				
		9487	....	....	....	....	....	....	....	....	S	S	S	S	D		
	2	9490	....	....	....	....	....	....	....	....	S	S	M	D			
		9514	....	....	....	....	....	....	....	S	S	S	D				
		9503	....	....	....	....	....	....	....	W	S	S	S	M	D		

Average time until signs appeared—8.3 days (8.3-4.1-4.2).
Signs appeared 4.2 days after probable exposure.

W—Weak; S—Signs; M—Moribund; D—Dead.

RESULTS

The groups of birds exposed to aërosols of NDV showed signs of the disease after three to four days, with the exception of 1 bird which failed to develop characteristic or typical signs. Respiratory involvement preceded the development of the characteristic nervous manifestations of ND. Sixteen susceptible birds were used to determine the potentiality of the virus to spread by the usual convection currents present in this room. This lot of birds started to show signs of the disease on the sixth to seventh day. The initial signs were weakness, râles, coughing, respiratory distress and, later, paralysis of the extremities and rhythmic spasms of the head and neck, or both. Some individuals showed the signs only after ten to eleven days. Fifteen of the 16 test birds died of the disease and the other was destroyed in the terminal stages of the disease. The results of two identical experiments (1 and 2) are summarized in table 1.

The presence of virus in the sampled air was determined by inoculation of the filter-washings in embryonating eggs. Allantoic fluids harvested from eggs which died were tested for hemagglutinative activity; only a few possessed significant activity, a titer of 10 or higher. Regardless of whether they were hemagglutinative or not, the allantoic fluids were inoculated into a second series of 10-day-old embryonating eggs. On this passage, the embryos died with few exceptions and hemagglutinative activity was induced by most of the allantoic fluids. The results are recorded in table 2.

When the results of initial virus isolation were compared, the use of gelfoam and the filter paper was found to be more efficient than the liquid impinger method for sampling the air for ND virus. The identification of virus in all instances was based both on lethality for the inoculated embryos and ability of the allantoic fluid from each embryo to produce agglutination of erythrocytes.

Newcastle disease virus was isolated from the expired air obtained from chickens on the third day after aërosol exposure, although allantoic fluids from the first em-

TABLE 2—Isolation of Newcastle Disease Virus from the Samples of Air from Cages Harboring Infected Chickens

Isolations Obtained on the First Passage in Embryonating Eggs

Days post-infection sample taken	Filter paper			Liquid impinger			Gelfoam		
	eggs inoc.	embryos dead	HA+	eggs inoc.	embryos dead	HA+	eggs inoc.	embryos dead	HA+
0*	16	0	0	8	0	0	8	0	0
1	16	3	0	8	0	0	8	0	0
2	16	1	0	8	0	0	8	0	0
3	16	5	0	8	2	0	8	0	0
4	16	5	3	8	1	0	8	5	4
5	16	2	1	8	2	0	8	1	1
6	16	2	1	8	0	0	8	1	0
No. samples from which isolations were made			3			0			2

Isolations Obtained by Repassage in Embryonating Eggs of Material from Embryos Dying on the First Passage

Days post-infection sample taken	Filter paper			Liquid impinger			Gelfoam		
	eggs inoc.	embryos dead	HA+	eggs inoc.	embryos dead	HA+	eggs inoc.	embryos dead	HA+
0*	0	0	0	0	0	0	0	0	0
1	12	0	0	0	0	0	0	0	0
2	4	0	0	0	0	0	0	0	0
3	20	13	6	8	8	8	0	0	0
4	20	16	13	4	3	2	20	20	20
5	8	6	4	8	6	5	4	4	4
6	8	4	2	0	0	0	4	4	4
No. samples from which isolations were made			4			3			3

*—Sample taken before exposure.

bryo passage of this material did not agglutinate red cells. On repassage in eggs, hemagglutination was demonstrated. Expired air from birds on the fourth day after aërosolization infected embryos and the virus could be demonstrated by the hemagglutination test, on the first passage, in a large proportion of the allantoic fluids from dead embryos. The proportion of embryo deaths in eggs which received the material obtained on the fourth day was higher than among those receiving material collected on the third, fifth, or sixth days.

Newcastle disease virus was isolated by the filter paper method on the third, fourth, fifth, and sixth days, by gelfoam on the fourth, fifth, and sixth days and by the liquid impinger on the third, fourth, and fifth days postexposure of chickens.

Virus could not be demonstrated in the samples of air taken from the cages in the isolation building before this experiment was started. Due to the death of most of the subjects, the sampling of air was not attempted subsequent to the sixth day of exposure.

DISCUSSION

The stage of the incubation period of ND appears to be an important factor in the dissemination of ND virus by means of aërosols. Normal susceptible birds showed signs of the disease as early as the third day and approximately all were affected on the fourth day after exposure to infective aërosols. Contact birds first showed signs of the disease after six days and on the average, at eight days. This suggests that the contact birds became infected on the third day or as soon as the principal birds began to show respiratory signs.

Sinha and associates[4] found that the concentration of NDV of six different strains was very high in lung tissue in chickens after aërosol exposure. Similar observations were reported by Hofstad[5] who inoculated four strains of NDV by several parenteral routes. That the virus was present to a high titer in the lungs of chickens on the third and fourth days is logical to the first finding of the virus in the samples of air from the cages of infected chickens on these days.

All susceptible contact birds in this experiment manifested signs of NDV within a short incubation period. This indicates that infection is not only possible through the air but that it occurs readily.

Various strains of NDV have produced a high concentration of virus in the tracheal exudate, saliva, and nasal secretions.[6] It is reasonable to assume that droplet nuclei may form from these materials and float in the air. As such, or with dust, the virus may be carried for considerable distances. The degree or extent of dispersion of NDV by coughing or sneezing obviously will depend upon such factors as quantity of the secretion, the concentration of the pathogen in the secretion, and upon the force and frequency of the sneezing. Loosli[7] believes that dust-borne pathogens may play a more important role in the transmission of some respiratory infections than does direct inhalation of infectious droplets or droplet nuclei. Although the relative importance of droplet nuclei and dust-borne particles as compared to other means of spread of virus infections is not known, infective droplets constitute a potential source of infection particularly in diseases of the respiratory tract. The experimental results of the present study and the reports by Delay *et al.*[2] indicate that aërosol transmission is probably a major means of spread of Newcastle disease among poultry.

If an epizoötic is initiated by air-borne infection, its continuance depends upon the maintenance of minimal or greater infectious concentrations in the air. The number of virus particles exhaled, the movement of air in the particular atmosphere, and the susceptibility of inhaling animals are probably the most important factors in this route of spread.

The results compiled in table 2 may be used to calculate approximate concentrations. Each day, 25 liters of air were sampled from the cages which harbored chickens in the incubative or active stages of ND. The samples were collected in 10 ml. of fluid. The distribution of the virus particles in such air samples can be estimated from the results of the gelfoam filtration studies. Ten milliliters of gelfoam suspension samples represented 25 liters of air. Each embryonating egg received 0.2 ml. or the equivalent of the quantity of virus present in 0.5 liters of air. The data in table 2 show that 5 of 8 embryos receiving the sample taken on the fourth day died on the first passage. Hence, on the basis of

Poisson distribution there were one to ten particles of virus present in 0.5 liters of sampled air, and approximately five to 50 particles in 2.5 liters of air. The respiratory intake of an adult chicken per minute was not found in a search of the literature, but compared on a weight basis with known rates for mammals the intake of chickens would be about 2.5 liters per minute. With each minute of exposure, the birds would be expected to inhale five particles of NDV. How long a susceptible chicken could breathe such air without becoming infected is unknown, but there is little doubt that such low concentrations of virus in air are readily infective. The calculated natural aërosol appears to contrast with 10^2-10^3 l.d.$_{50}$ virus concentrations in artificial aërosol clouds employed particularly in vaccination in most studies (personal communication). Since it is unlikely that the porosities of the gelfoam filter and the filter paper are small enough to hold back passage of all particles of the Newcastle disease virus, the artificial aërosol may not differ as much from the natural as it appears. Accurate determination of the number of particles in a measured volume of air in an infected area must await further refinements of methods and the compilation of more data.

SUMMARY

Newcastle disease virus was recovered from the air in the immediate vicinity of infected chickens on the third, fourth, fifth, and sixth days following simulated natural exposure. The air was sampled by filtration through gelfoam® or filter paper or by passage through a liquid impinger which contained normal allantoic fluid. The criteria of virus recovery were the death of embryos inoculated with the sample material and the demonstration that the allantoic fluid of these embryos possessed specific hemagglutination activity. Gelfoam yielded more first passage isolations of Newcastle disease virus, but all of the three procedures were efficient as indicated by the number of isolations obtained on repassage in eggs.

Natural aërosols produced by infected chickens appear to contain but few infective particles in a liter of air.

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Propagation of a Mixed Culture of a Chronic Respiratory Disease Agent and Newcastle Disease Virus in Chicken Embryos

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REPORTS were first received in 1950 calling attention to a new respiratory disease entity which was reported to be fairly widespread in the broiler-raising areas of the eastern seaboard. It was reported to be characterized by rather slow spread through affected flocks and manifested by respiratory distress, *viz.*, sneezing, some gasping, and audible râles. The principal findings at necropsy were fibrinous or caseous exudate on the linings of the air sacs and mucous or caseous curds of exudate in the lumen of the trachea and primary bronchi. It was the conspicuous involvement of the air sacs which gave rise to the popular term "air sac infection." By 1952, major losses in broiler production were ascribed to this condition.

Although it has not been fully determined that the etiology of so-called air sac infection in broilers is the agent originally described by Nelson [8] and later propagated in chicken embryos by Delaplane *et al.*[4, 5] from cases of a chronic respiratory disease in adult fowl, evidence favors this belief. The frequent occurrence of air sac infection as a complication following the use of live virus Newcastle disease vaccines has suggested that its transmission might be associated in some manner with this vaccination procedure. The possibility of contamination of serials of live virus Newcastle disease vaccine with the agent of chronic respiratory disease (CRDA) has been suggested.[3] To investigate this possibility, techniques for the examination of vaccines for a CRDA needed to be developed.

In the experiments described in this paper, the aforementioned problem was approached by deliberately contaminating cultures of two different strains of Newcastle disease virus (NDV) with a strain of CRDA. These studies resolved a technique for the demonstration of the presence of CRDA in NDV cultures.

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MATERIALS AND METHOD

Chronic Respiratory Disease Agent.—The culture of CRDA used in this study was supplied to the Bureau of Animal Industry by Dr. Henry Van Roekel, University of Massachusetts, in July, 1952. It was identified as CR-B-seventeenth passage. A stock culture of this agent was prepared by collecting allanto-amniotic fluids seven days after inoculation of 7-day-old chicken embryos into the yolk sac. Aliquots of this material were stored in rubber-stoppered vials in a dry ice chest to be used as needed.

Newcastle Disease Virus, B_1.—The B_1 strain of NDV[6, 7] was from a stock culture maintained in the laboratories of the Bureau. It is a strain of virus used for commercial production of intranasal Newcastle disease vaccine.

Newcastle Disease Virus,[1] California 11914.—This is a strain of NDV which is moderately virulent for chickens of all ages. It was one of the earlier isolations of NDV to be made in this country and has been widely used in the study of Newcastle disease.

Embryonating Eggs.—The eggs used in these experiments were laid by the Bureau's experimental flocks. These flocks are routinely vaccinated via the wing-web with live virus Newcastle disease vaccine while on the growing range.

Newcastle Disease Antiserum.—The Newcastle disease antiserum (BAI lot 8) was prepared in February, 1952, from serial bleedings by cardiac puncture of mature roosters which had survived exposure to intramuscular injection of the California strain 11914 NDV. It was made sterile by filtration through a Mandler filter No. 8. Merthiolate to a concentration of 1: 5,000 was added as a preservative. In serum-neutralization tests, 0.05 cc. of serum has regularly neutralized in excess of 100,000 embryo $l.d._{50}$ of NDV.

Inoculation Technique.—Injections were preceded by painting the shell over the air cell with tincture of iodine. Then with a sterile bit in a vibro-tool a hole was drilled through the shell over the air cell. All embryos were inoculated with 0.1 cc. of inoculum. Inoculations into the allantoic cavity of 10-day-old embryos were made by using small hypodermic needles, ¾ in. long, and intra-yolk sac inoculations of 7-day-old embryos were made with needles 1 in. long. After inoculation, the holes in the shells were closed with sealing wax.

EXPERIMENTAL DATA

Characterization of Strain B CRD Agent.—The first step in the proposed experiments was to be-

come thoroughly familiar with the propagation pattern in chicken embryos of several strains of CRDA. The strain B was selected for the experiments because of its pathogenicity for embryos. It produced approximately 75 per cent mortality in chicken embryos when inoculated into the yolk sac of 7-day-old embryos. Observations were made to the eighteenth day of incubation. In limited exploration, it appeared that inoculations into the allantoic cavity gave a slightly lower rate of embryo mortality. The variation in the mortality pattern obtained in 15 trials with aliquots of the stock culture appears in table 1. The greatest mortality occurred on the fifth, sixth, seventh, and eighth days postinoculation. Random samples of allanto-amniotic fluids were collected during these trials and tested for hemagglutination. None produced hemagglutination* of chicken erythrocytes.

An infectivity titration of the strain B CRDA indicated that 1 cc. of allanto-amniotic fluids contained somewhat less than 1,000,000 embryo infective doses (table 2). In one experiment, it was determined that lot 8 of the Newcastle disease antiserum, containing merthiolate as a preservative, had no suppressive or deleterious effect on the growth of CRDA when mixed with the agent prior to injection into eggs. This information was necessary to assure that in the proposed neutralization of NDV in CRDA-contaminated cultures of NDV, the merthiolate would have no adverse effect on the CRDA.

Van Roekel *et al.*[10] have reported the production of an abscess-like process in the region of the articulations of the appendages of chicken embryos by 16 of 53 isolations of CRDA. The strain B CRDA produced inflammatory enlargement and prominent abscesses of the articulations of the legs and wings in about 10 per cent of the dying embryos. These lesions were observed most commonly in embryos dying on the seventh and eighth days postinoculation. Actually, the lesions were more prominent in the fresh specimens than in the accompanying photographs (fig. 1) made from formalin-fixed specimens in which the abscesses have tended to shrink. Histological preparations show that these lesions are abscesses composed of aggregations of heterophils. Erosion of the articular surfaces of the joint cavity was evident also. Focal necrosis of the liver was observed both grossly and histologically in a small percentage of the dead embryos.

In subsequent experiments, the occurrence of the characteristic mortality pattern, the observance of the lesions of the articulations in a small percentage of the dying embryos, and the absence of hemagglutination by the allanto-amniotic fluids were taken as indicative of the presence of the strain B CRDA.

The B_1 Strain of NDV.—Intranasal live virus Newcastle disease vaccine is commonly produced from the B_1 strain of NDV. Experience in this laboratory has been that the B_1 strain of NDV seldom produces mortality in 10- to 12-day-old embryos, the age commonly used for NDV propagation and vaccine production. It is best detected by the hemagglutination test.[2] In 7-day-old embryos inoculated intrayolk sac, it was observed that this virus produced a mortality pattern not unlike that which has been recorded for the strain B CRDA in this paper. Even though the mortality pattern of the B_1 NDV 7-day-old embryos was similar to that of the CRDA, the specific hemagglutination-inhibition test for Newcastle disease and the absence of articular abscesses in the dead embryos did serve to differentiate it from the CRDA.

Propagation of a Mixture of Strain B CRD

* At the twenty-fifth annual meeting of the Northeastern Conference of Laboratory Workers in Pullorum Disease Control at Amherst, Mass., June, 1953, R. E. Jacobs *et al.* described a hemagglutination test for air sac infection. They prepared an antigen by growing a strain of CRDA *in vitro* in liquid medium.

TABLE 1—Mortality Pattern Obtained by Intrayolk Sac Inoculation of the Agent of Chronic Respiratory Disease in 15 Lots of 7-Day-Old Chicken Embryos

No.* inoculated	Number of embryos dying and day of death after inoculation									No. alive on day 18
	(day) 3	4	5	6	7	8	9	10	11	
10					4		1	2	1	2
12				7		3	1			1
36			13	5		13				5
33		2	4	6	4			9	2	6
40				8	7	2	4	1		18
40				4	3				17	16
20				5	4		2	1		8
17			13	3	1					0
19		13	4	1	1					0
19				4	2	2	2	3		6
20				6	2	4	3	2		3
17				6	1	1	5			2
15					6	4	2	1		2
14					5	1	1	3	1	3
20				3	1	4	1			11
332	0	15	34	58	41	34	22	22	21	83
Percentage		4.5	10.2	17.5	12.3	10.2	6.6	6.6	6.3	25.0

* Embryos dying the first 48 hours after inoculation are not included.

Agent and B_1 NDV in 7-Day-Old Embryos Inoculated Intrayolk Sac. — Fifty, 7-day-old embryos were inoculated into the yolk sac with a mixture composed of equal parts of a 1: 1,000 dilution of B_1 NDV (allanto-amniotic fluids) and a 1: 10 dilution of the CRDA (allanto-amniotic fluids). Embryo deaths occurred as follows: first day, 3; second day, 3; third day, 4; fourth day, 22; fifth day, 12; seventh day, 3; and on the eighth day, the remaining 3. Individual samples of allanto-amniotic fluids were collected from 12 randomly selected embryos which had died on the second, third, and fourth days postinoculation. Eight of them had hemagglutination titers of 1: 320 or higher. The undiluted allanto-amniotic fluids from these embryos were pooled and injected into another group of 7-day-old embryos.

Embryo mortality for the second passage of the mixed culture was as follows: first day, 1; third day, 6; fourth day, 2; and fifth day, 1. Subsequently, three more passages were made in 7-day-old embryos. Beginning with the third passage, the virulence of the mixed culture increased so that nearly all of the embryos died on the second and third days postinoculation. The allanto-amniotic fluids collected from each passage had a hemagglutination titer of 1: 320 or greater.

In the meantime, the pool of allanto-amniotic fluids from the first passage was diluted 1: 1,000, mixed with equal parts of undiluted Newcastle disease antiserum, and injected into the yolk sac of 40, 7-day-old embryos. It was reasoned that an allanto-amniotic fluid dilution of 1: 1,000 would be about the minimum dilution which would regularly assure that all of the NDV would be neutralized by the antiserum. The first embryo deaths occurred six days postinoculation and by the eighteenth day of incubation 24 of the 40 embryos had died. Several of the dead embryos had abscesses of the hocks. Focal necrosis of the liver was also observed in several embryos. Pooled allanto-amniotic fluids gave a hemagglutination titer of 1: 160. It was evident that not all of the NDV had been neutralized.

The allanto-amniotic fluids collected from the second passage of the mixed culture, as with the first passage, were diluted 1: 1,000, mixed with equal parts of Newcastle disease antiserum, and inoculated into 20, 7-day-old embryos. Embryo deaths occurred principally on the sixth and seventh days after this inoculation. No enlargement of the articulations of the appendages of the dead embryos was observed. However, the allanto-amniotic fluids from these embryos were negative to hemagglutination.

This step was repeated with the allanto-amniotic fluids of each subsequent passage diluted 1: 1,000, neutralized in an equal volume of undiluted Newcastle disease antiserum, and then injected into 20, 7-day-old embryos. The third passage material after neutralization yielded several embryos with abscessed hocks and several with visible areas of necrosis in the liver. Pooled allanto-amniotic fluids collected from the dead embryos were negative to hemagglutination. Similar observations were made for the neutralized material of both the fourth and fifth passages.

The experiment was not carried beyond the fifth passage. It was reasoned that injection of 0.1 cc. of inoculum into the yolk sac represents a dilution of about 1: 180, considering the weight of the yolk as 18 Gm. Five chicken embryo passages without any multiplication of CRDA would be equivalent to diluting the original material nearly 200 billion times—a million times greater than the agent's infectivity titer.

Thus, it was demonstrated that NDV and the CRDA simultaneously propagate when inoculated into the yolk sac of 7-day-old chicken embryos.

Propagation of a Mixture of Strain B CRD Agent and B_1 NDV in 10-Day-Old Embryos Inoculated via the Allantoic Cavity.—Cognizant that NDV is commonly propagated in 10- to 12-day-old embryos by inoculation into the allantoic cavity, it was considered that a practical aspect of this problem would be to determine whether a mixed culture of B_1 NDV and strain B of the CRDA could be carried through serial passages in embryos of this age by this route. As in the previous experiment, cultures of strain B CRDA and B_1 NDV were mixed and injected into the allantoic sac of 10-day-old embryos. After seventy-two hours of incubation, the time when embryonic tissues are usually harvested in commercial production of intranasal Newcastle disease vaccine, allanto-amniotic fluids were collected from both living and dead embryos, and inoculated undiluted into another group of 10-day-old embryos. This

TABLE 2—Embryo Infectivity Titration of the Agent of Chronic Respiratory Disease by Intrayolk Sac Inoculation of 7-Day-Old Chicken Embryos

Dilution	Number of embryos dying and day of death after inoculation									No. alive on day 18
	(day) 3	4	5	6	7	8	9	10	11	
10^{-1}*			1	1	2			3	1	2
10^{-2}				2				3		2
10^{-3}		1	1	1	1				1	2
10^{-4}		1	2	2	1			3		0
10^{-5}			1	2						5
10^{-6}								1		9

* Ten embryos were inoculated per dilution. Embryos dying the first 48 hours after inoculation are disregarded.

was repeated for five passages. During these passages the embryos dying the first twenty-four hours after inoculation were discarded.

The allanto-amniotic fluids of each passage were tested by hemagglutination test for the presence of NDV. Titers of from 1: 320 to 1: 640 were obtained for each sample, thus indicating a significant quantity of NDV present in each passage.

Neutralization of the NDV to demonstrate the presence of the CRDA was only performed on the allanto-amniotic fluids collected from the fifth passage of the mixed culture. These fluids were diluted 1: 10,000 since, in the previous experiment, a 1: 1,000 dilution of the allanto-amniotic fluids did not always result in the NDV being neutralized by the antiserum. They were then added to an equal quantity of undiluted Newcastle disease antiserum and injected into the yolk sac of 20, 7-day-old embryos. The mortality pattern among these embryos was typical for CRDA. Several of the dead embryos had typical abscesses on the hock joints. A pool of allanto-amniotic fluids from these embryos had no hemagglutinating activity, thus indicating that the NDV had been successfully neutralized.

The experiment demonstrated that a culture of B_1 NDV contaminated with strain B CRDA might be carried indefinitely, and unsuspectingly, by the

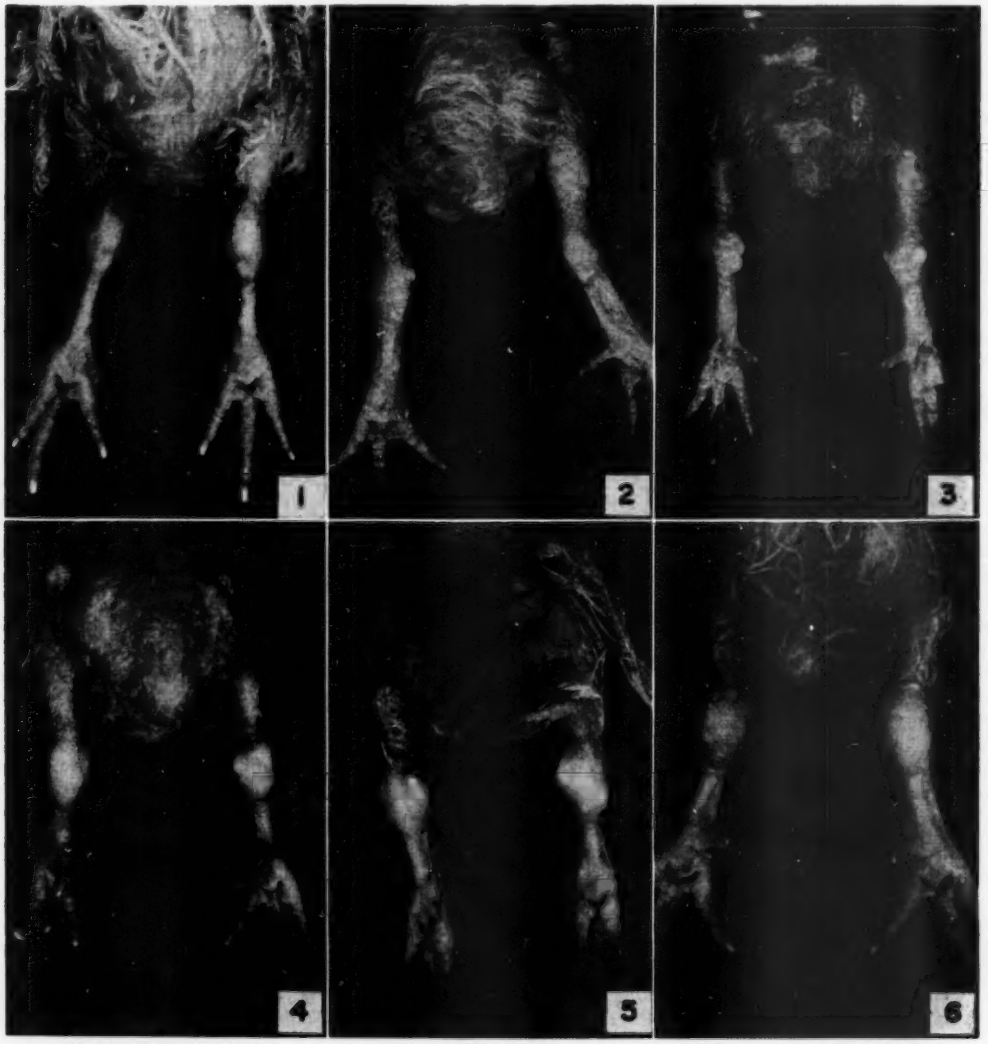

Fig. 1—Lesion produced by the chronic respiratory disease agent: (1) Normal 14-day-old chicken embryo; (2) single abscess on the medial surface of the left hock; (3 to 5) multiple abscesses on both the medial and lateral surfaces of both hocks; and (6) inflammatory enlargement of the hocks without abscess formation.

procedures normally followed in working with NDV.

Propagation of a Mixture of Strain B CRD Agent and the California 11914 NDV in 10-Day-Old Embryos Inoculated via the Allantoic Cavity.—Another experiment was conducted using the California strain 11914 NDV, a relatively virulent virus, mixed with the strain B CRDA, as in the previous experiments, and inoculated into the allantoic cavity of 10-day-old embryos. This mixed culture was carried through five passages. In this experiment, each passage was automatically terminated in forty-eight hours because of the death of the embryos from the NDV. By diluting the allanto-amniotic fluids of the fifth passage 1: 10,000 and mixing with undiluted Newcastle disease antiserum, the presence of the CRDA was readily manifested by the characteristic embryo lesions and mortality pattern and by the absence of hemagglutination of the allanto-amniotic fluids. This experiment demonstrated that a NDV culture having greater virulence than the B_1 NDV might also carry an unsuspected contamination of CRDA.

DISCUSSION

The experiments set forth in this paper show that when a culture of NDV is deliberately contaminated with a culture of the CRDA and inoculated into chicken embryos by either the intrayolk sac or the intra-allantoic cavity route of inoculation, both agents propagate. It is demonstrated that a CRDA-contaminated culture of NDV can be carried through several embryo passages without the contamination being suspected. Van Herick and Eaton,[9] reporting the spontaneous appearance of a pleuropneumonia-like organism in egg-propagated virus cultures with which they were working, suggest the possibility that eggs may be laid carrying such contamination. More recently, Van Roekel *et al.*[10] have reported evidence that chronic respiratory disease of poultry can be transmitted via the egg. Hence, the finding reported in this study bears considerable significance, particularly to the production of embryo-propagated live virus vaccines.

The demonstration that NDV can be neutralized from a mixed culture leaving only the CRDA has practical application. It is a procedure that is quite applicable for the examination of live virus Newcastle disease vaccines for contamination with CRDA.

SUMMARY

1) It is shown that two cultures of Newcastle disease virus (NDV) of different virulence, each deliberately contaminated with a strain of the agent of chronic respiratory disease (CRDA), remained contaminated through five serial passages in chicken embryos. Evidence of contamination with the CRDA was not manifested.

2) A technique is described for the demonstration of the CRDA in such a mixed culture. It consists of neutralizing the NDV with Newcastle disease antiserum and then inoculating the neutralized material into the yolk sac of 7-day-old chicken embryos. In the experiments conducted, the CRDA was manifested by characteristic embryo lesions and mortality pattern and by the absence of hemagglutination by the allanto-amniotic fluids.

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A New Record for Gapeworm (*Cyathostoma Bronchialis*) Infection of Domestic Geese in North America

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IN THE LATE autumn of 1951, the writers had an opportunity to examine some domestic geese which were found to be heavily infected with *Cyathostoma bronchialis*. Since this nematode does not appear to have been reported previously from domestic geese on this continent, the following observations are recorded.

HISTORY OF OUTBREAK

The infected geese were raised in the vicinity of Duluth, Minn. The course of the disease extended over a period of five months during which time the owner estimated an over-all morbidity of about 80 per cent with a death loss of about 20 per cent. After initial symptoms of respiratory distress were noticed, some birds died within a few days whereas others lingered for several weeks. The affected birds showed extreme respiratory distress, throwing back their heads and gasping for breath. The symptoms exhibited were quite similar to those of laryngotracheitis. Growth of individuals that recovered appeared to be retarded.

REVIEW OF LITERATURE

In 1928, Cram [1] recorded gapeworm infections in wild geese raised in captivity in north central Illinois. Young Blue, Cackling, Snow, and Canada geese were found to be infected with gapeworms; the mortality was high in birds ranging from 10 to 14 days of age. The species of Cyathostoma infecting these wild geese appeared to have a restricted host range, since large numbers of chickens, turkeys, and wild ducks of several varieties ranged over the same ground with no evidence of harboring gapeworms.

Although species of Cyathostoma have been reported as being widespread in domestic geese in Europe, the only report of members of this genus in water birds on this continent, prior to that of Cram,[1] was in a swan in Ithaca, N. Y., in 1920. The specimen from the swan was a single female in a poor state of preservation. In a review of this genus in 1925, Chapin [2] considers this specimen to resemble *C. bronchialis*.

In 1932, Christenson [3] reported *C. bronchialis* from the Canada goose *(Branta canadensis)*, the Bean goose *(Anser fabalis)*, and the Blue goose *(Chen caerlescens)*. These geese were raised in captivity in Minnesota for exhibition purposes.

DESCRIPTION OF CYATHOSTOMA BRONCHIALIS

Although the nematodes examined were ruptured and not in a good state of preservation, the following observations were recorded.

Male.—The length of several males was 9.5 to 10.0 mm., with a maximum width of 0.25 to 0.328 mm. The buccal capsule was somewhat wider than deep; internal diameter, 0.09 to 0.13 mm.; depth, 0.14 mm.; external transverse diameter, 0.13 to 0.17 mm. Usually six but occasionally seven triangular buccal teeth of equal size were present. The esophagus was 0.560 to 0.570 mm. in length and clavate in shape. The spicules were long, thin, and filamentous, varying from 0.54 to 0.70 mm. in length. The tips of spicules fimbriate, appearing united and slightly incurved. The males are not necessarily joined in permanent copula.

Female.—The length of several females was 16.0 mm. to 26.0 mm., with a maximum width of 0.75 to 0.86 mm. The buccal capsule was slightly broader than deep; internal transverse diameter, 0.33 to 0.42 mm.; depth, 0.325 mm.; external diameter, 0.45 to 0.525 mm., and there were six to seven buccal teeth of about equal size. The esophagus was 1.0 to 1.14 mm. in length and clavate in form. The vulva with fairly prominent lips was situated in the vicinity of the posterior part of the anterior third of the body. The tail was acute and about 0.075 mm. in length. Eggs were 0.068 to 0.085 mm. in length and 0.043 to 0.058 mm. in width. The eggs in the region of the vulva showed a slight operculum at one end.

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TABLE 1—Range in Size (in mm.) of 2 Male and 3 Female Specimens of Cyathostoma Bronchialis

	Length	Maximum width	Number of teeth	Buccal capsule: Depth	Buccal capsule: Internal diameter	Buccal capsule: Wall thickness	Length of esophagus	Spicule length	Ova
Males.....	9.5 to 10.0	0.250 to 0.328	6	0.14	0.090 to 0.130	0.040	0.560 to 0.570	0.540 to 0.700	.....
Females..	16.0 to 26.0	0.750 to 0.860	6 to 7	0.325	0.330 to 0.420	0.03 to 0.1	1.0 to 1.14	.....	0.068 to 0.085 by 0.043 to 0.058

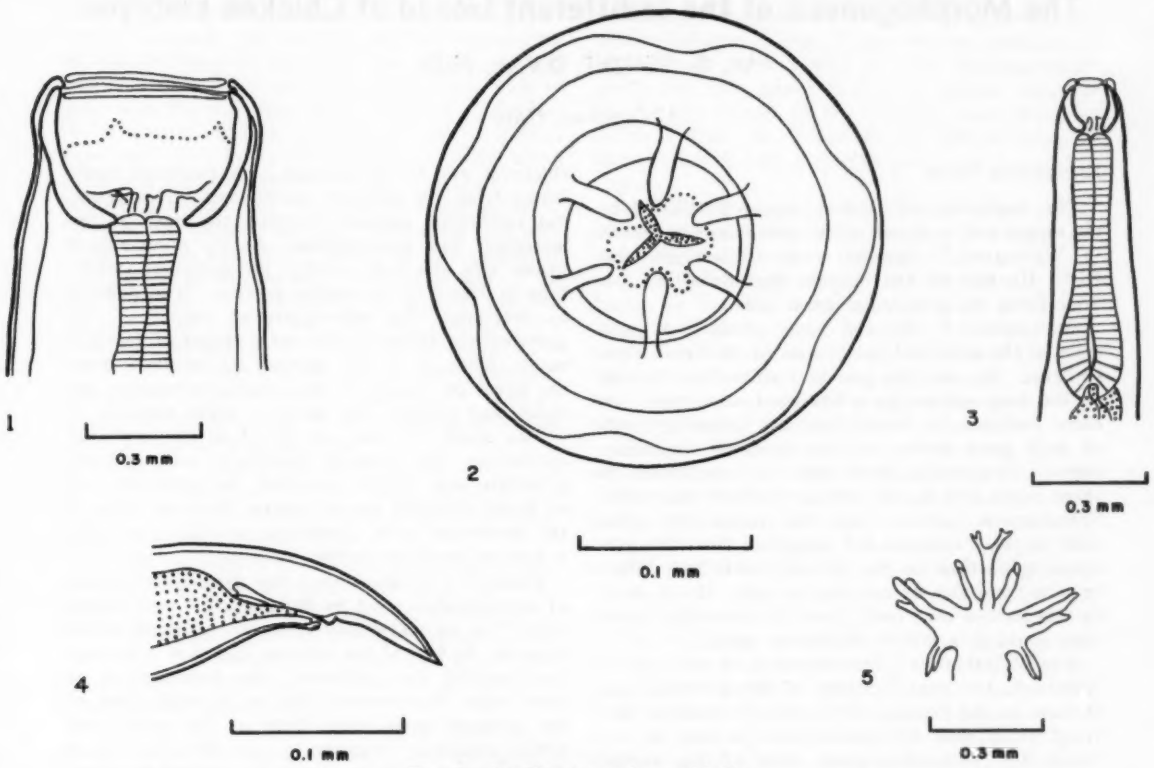

Fig. 1—Cyathostoma bronchialis.

(1) lateral view of head of female;
(2) "enface" view of head of female;
(3) lateral view of head and esophagus of male;
(4) tail of female;
(5) bursa of male.

COMPARISON OF CYATHOSTOMA SPECIES

On initial examination, it was thought that these helminths were likely to be a species of Syngamus; however, since the males were not firmly united in copula, they were considered to be representatives of the genus Cyathostoma. In comparison with those listed by Cram,[4] these specimens did not conform exactly to any one specific description. With a few minor differences in measurement, the species conforms in general to Chapin's[2] description of *Cyathostoma bronchialis*. The specific measurements of structures examined showed considerable size range proportionate to the size of the parasite; such variation was also recognized by Chapin.

In comparing *C. bronchialis* with *C. brantae* from the goose as described by Cram,[1] the former varies considerably in the shape of teeth at the base of the buccal capsule, in the presence of prominent vulval lips, in the branching of the tips of the dorsal ray of the bursa, and in the absence of a gubernaculum.

SUMMARY

A species of gapeworm considered to be *Cyathostoma bronchialis* has been reported from domestic geese in Minnesota. This helminth infection appeared to be primarily responsible for extensive losses among the goslings of this flock. The morbidity in the outbreak was about 80 per cent with a mortality of about 20 per cent. This species has been previously reported from the Canada goose, Blue goose, and Bean goose in Minnesota. It seems probable that this infection was introduced to the domestic flock by wild birds which may have associated with the domestic birds while resting during their annual migration flight.

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The Morphogenesis of the Indifferent Gonad of Chicken Embryos

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LITERATURE CITED

The beginning of modern research relative to the origin and products of the sex glands appeared in Waldeyer's[40] famous work "Eierstock und Ei." He was of the opinion that definitive ova arise from the primordial germ cells.

Schmiegelow[30] observed large primordial germ cells in the germinal epithelium of young chicken embryos. He saw the germinal epithelium limited on its deep surface by a basement membrane. In older embryos, he found that anastomosing cords of cells grow down into the subjunct medullary tissue. Primordial germ cells are contained in these cords and in the stroma between the cords. Schmiegelow believed that the primordial germ cells in the stroma do not emigrate from the germinal epithelium in the cellular cords but differentiate from the mesenchyme *in situ*. It appeared to this author that both kinds of primordial germ cells could give rise to definitive eggs.

Von Mihalkowics[37] first observed, in embryos of Amniotes, two proliferations of the germinal epithelium in the female. This author's opinion differed from that of Schmiegelow in that he believed the primordial germ cells of the sexual cords, as well as those seen in the germinal epithelium, are of peritoneal origin.

Authors differ widely in their opinion concerning the origin of the sexual cords in birds. Janosik[19] believed that these cords are ingrowths of the germinal epithelium. Semon[31] and Hoffmann[18] believed that they arise as outgrowths of the capsules of renal corpuscles. Semon states that they may arise from the neck of the wolffian tubules.

Prenant[24] studied the evolution of the gonad, particularly the origin of the primordial germ cells. In the 3½-day chicken embryo, he observed the genital ridge is covered with the elongated cells of the germinal epithelium. In this germinal epithelium and in the mesenchyme which lies beneath it forming most of the genital ridge, he observed large primordial cells. No mitotic figures were found among these primordial germ cells, yet they increased in number; he concluded that they were produced by differentiation of the cells of the germinal epithelium.

Hoffmann[18] found that primordial germ cells exist before the appearance of the germinal epithelium and gonad. He showed that the definitive ova are in a direct line of descent from the true primordial germ cells.

The germinal epithelium is differentiated on the fourth day, according to Lillie,[21] by its greater thickness and by the absence of a basement membrane from the adjacent peritoneum. The germinal epithelium appears between the base of the mesentery and mesonephros. As the mesonephros grows into the body cavity, the germinal epithelium is drawn to its median surface. It is difficult to determine the anteroposterior extent of the germinal epithelium in the early stages of development. Lillie is of the opinion its origin is near the point of origin of the omphalomesenteric arteries and extends over seven or eight somites.

Two kinds of cells are found in the germinal epithelium—the ordinary peritoneal cells and the primitive ova. Lillie described the primitive ova as being typically round, several times as large as the peritoneal cells, possessing a clear cytoplasm, a nucleus, and one or two nucleoli.

Firket[11,12] believed that the cytological aspect of mitochondria used by Rubaschkin[28] and Tschaschin[35] is an unreliable criterion of germ cells; however, he was of the opinion that it is a convenient method for following the development of these cells. He observed that in the chick most of the primary germ cells, both of the ovary and testis, eventually degenerate, and the majority of definitive ova or spermatozoa are derived from the epithelial tissues of the sex glands. These constitute a second generation of germ cells and are called secondary germ cells. On the other hand, Firket saw no reason why "certain of the primary germ cells might not produce some of the definitive cells, since it was impossible to distinguish between primary and secondary cells."

Von Berenberg-Gossler,[36] working with birds, reported recognizable germ cells differentiating from the genital anlage coincident with its development. He believed that large cells, appearing before the gonads developed, are not germ cells but cells participating in a late formation of mesenchyme.

Fell[10] studied the gonads of 8 birds representing various stages in sex-reversal from female to male. This author found germ cells of the opposite sex proliferating from the peritoneal epithelium of the degenerating ovary. The sex cords gave rise to seminiferous tubules and functional sperm.

Swift[32,33,34] published three papers describing researches on the germ cells of the chick. He observed the germ cells arise in a region anterior and anterolateral to the primitive groove and at the margin of the area pellucida. These cells are first seen between the entoderm and ectoderm. When the mesoderm appears in this region, these cells enter the tissue and, later, the blood vessels. According to Swift, the primordial germ cells of his observation are identical with the "entoder-

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mal wonder cells'' described by Dantschakoff.[5] Swift disagreed with the latter in that he observed no degeneration of the cells which, Dantschakoff believed, eliminated all primordial germ cells so that they did not participate in gonad formation. Swift concluded that practically all primordial germ cells do persist, and are carried by the blood stream to all parts of the embryo. At the twenty-second to the twenty-ninth somite stage of development, the primordial germ cells take up residence in the splanchnic mesoderm and coelomic epithelium. About the sixth or seventh day of incubation the cells pass into the gonad, and the extragerminal tissues arise as proliferated cords from the epithelium.

Nussbaum,[23] Hoffmann,[18] Kiebel and Abraham,[20] Rubaschkin[27] and von Berenberg-Gossler[38] have all described the germ cells in the splanchnopleure of 22 to 23 somite bird embryos.

Woodger[41] studied the origin of the germ cells of the fowl by means of their Golgi bodies. It was possible with this technique to distinguish between germ cells and somatic cells.

Willier[39] identified primordial germ cells extra-embryonically and determined whether they were essential for gonadal development. Evidence is presented to show that in the chick no germ cells develop in the absence of primordial germ cells. When the area pellucida is isolated from all germ cells, no gonads develop.

Richards, Hulpieu, and Goldsmith[26] investigated the history of the germ cell in a series of chicken embryos from the beginning of incubation to 180 days after hatching. These authors find that the early development is entirely in accord with that described by Swift.[32, 34]

Goldsmith[15] first saw primordial germ cells in the embryo during the primitive streak stage at the outer edge of the proamnion, anterior, and anterolateral to the head fold. Up to the 33-hour stage of incubation, the primordial germ cells are found only in extra-embryonic tissues. These findings are in agreement with Dantschkoff[5] and Swift.[32] From 33 to 40 hours of incubation, the primordial germ cells are widely distributed throughout the embryo and blood stream. The cells gradually take up residence in the developing germinal epithelium. By the sixth or seventh day, sex could be determined accurately. No widespread degeneration occurred among the primordial germ cells of either sex.

Matsumota[22] stated that the primordial germ cells are first found in the posterior portion of the primitive streak and primitive plate in embryos of 10 hours of incubation.

The germ cell crescent of the blastoderm has been excised by Reagan,[28] irradiated with ultraviolet by Benoit,[2] and destroyed by an electric cautery by Dantschakoff *et al.*[7] Histological examination of blastoderms so treated and incubated for two or more days showed the complete absence of germ cells from the gonadal areas.

Willier[39] isolated portions of chick blastoderms at certain critical stages in the development of the primordial germ cells. These isolated portions of blastoderms were tested on the chorioallantoic graft for their power to form a gonad. Regardless of the nature of the isolated piece from the pellucid area, the gonad which differentiated is sterile, forming no male-like sex cords or ovarian cortex.

Lillie[21] has observed that sexual cords appear within the gonad on the fifth day of incubation. According to Semon,[31] the nature of the gonad may be detected on the fifth or sixth day of incubation by the fact that the right ovary is already much smaller than the left. Swift[33] stated that when the chicken embryo has developed for 6½ days, the formation of the sexual cords of first proliferation ceases, and the sex of the embryo can then be definitely determined.

Willier[39] presented evidence that the gonad, during the morphologically indifferent period of development, is specifically organized with respect to sex, and also with respect to laterality in the case of female embryos. This specific organization is already fixed in the gonads of 4-day embryos, which is the approximate time of origin of the genital ridge.

The origin of asymmetry in the reproductive system of birds has been referred to and studied by various workers. Firket,[11] in studies on the organogenesis of the sex glands of the domestic fowl, mentioned the unequal distribution of primordial germ cells in the 4¾-day embryonic sex glands. Swift[33] observed that at 90 hours of incubation, the left gonad is larger than the right. Swift found more germ cells on the left than on the right side and believed that occurs because the left gonad, from its first appearance was larger in size. Dantschkoff[6] observed that the the twenty-fourth to twenty-sixth somite stage of development the left gonad seldom contains less than twice and generally three to five times as many germ cells as the right gonad.

Witschi[40] concluded that the asymmetry in the gonads of birds was the result of a ''primary, hereditarily fixed deficiency of the right cortical inductor.'' This deficiency was thought to ''express itself in decreased attraction upon the primordial germ cells, especially during the phase of their redistribution in the early part of the third day.''

MATERIALS AND METHODS

All embryos and chickens used in this study were of a pure single comb, White Leghorn stock maintained by the Department of Poultry Husbandry at Iowa State College. Throughout the study, the fertile eggs were obtained from the same group of hens.

The study was made from a series of 420 embryos, ranging from 12 hours of incubation through 165 hours of development.

The gonads in the developing embryos were fixed in Bouin's or Helly's fluid. All tissues were

embedded in Altmann's paraffin mixture and serially sectioned at 6 to 8 μ. Several stains were employed to show the various structures of the gonads. Hematoxylin and eosin were used as a routine stain. Iron hematoxylin was admirably suited to the display of cytological structure in those tissues fixed with Helly's fluid. Mallory's connective tissue stain was employed after Helly's fluid in order to show the connective tissue elements of the gonads.

TABLE 1—The Number and Distribution of the Primordial Germ Cells in the 96-Hour Chicken Embryo

No. of embryos	Germ cells counted	Right gonad	Left gonad	Cells in left gonad (%)
1	700	181	519	74.14
1	626	149	476	76.16
1	908	304	604	66.52
1	852	285	567	66.55
Total	3,085	919	2,166	70.21

OBSERVATIONS

The primordial germ cells make their appearance in from 16 to 24 hours of development. These cells continue to form and to accumulate at their site of origin in the space between the ectoderm and entoderm. With the appearance and growth of the mesoderm the primordial germ cells must enter this germ layer. Much of the vascular system has started to develop in the mesoderm, thus distributing the germ cells to all parts of the vascular area and embryo. The germ cells remain distributed until some time between the 40- and 45-hour stage of development, at which time they disappear from the vascular system. It is during this period that the primordial germ cells enter the splanchnic mesoderm of the gonadal ridge.

Between 90 and 96 hours, the urogenital ridge appears on the medial surface of the wolffian body. This period marks the beginning of the indifferent stage in the development of the gonads, since the urogenital ridge is similar in all embryos whether they are destined to become male or female. The anterior half of the wolffian body contains the sexual elements which will become the gonads. Just posterior to the gonad, there is a nonsexual region of the wolffian body. Posterior to the entire wolffian body, the urogenital ridge is found to contain only the wolffian and mullerian ducts.

At 96 hours of development, the urogenital ridge is attached to the medial surface of the wolffian body and between that organ and the base of the mesentery. At this early stage, it is difficult to determine the anteroposterior extent of the germinal epithelium. It seems to begin at some point near the origin of the omphalomesenteric arteries and extends posteriorly for a distance of eight somites. The ridge consists of a single layer of thickened peritoneal epithelium (commonly called the germinal epithelium) and some underlying mesenchymal connective tissue. The germinal epithelium is composed of two kinds of cells, the ordinary peritoneal cells and the primordial germ cells. The germ cells are round and larger than the peritoneal cells. The germ cells at this time have a diameter of from 8 to 10 μ; the diameter of the peritoneal cells is from 4 to 5 μ. The cytoplasm is clear in the germ cells, and the nucleus contains one to two nucleoli. These cells are sharply distinguishable from the peritoneal cells.

The unequal distribution of the germ cells in favor of the left side is now definitely established. Prior to this time, the germ cells were rather evenly distributed between the right and left gonad. Of 3,085 germ cells counted from 4 embryos, 919 were present in the right gonad, and 2,166 were found in the left (table 1). Those germ cells present in the mesentery and the splanchnopleure of the right and left side were not counted.

At 110 hours of development, the germinal epithelium has increased in thickness to from three to five cell layers. At the same time, the underlying mesenchyme has also increased.

After 120 hours, the germinal ridges or indifferent gonads have increased in size. Their length varies from 1.5 mm. to 2.0 mm. and their thickness is approximately 0.50 mm. for the left and 0.30 mm. for the right (fig. 1). Growth of the wolffian body into the abdominal cavity has crowded the indifferent gonads toward the base of the mesentery. The gonads now rest on the ventral medial surface of the mesonephros in the space between the mesonephros and the mesentery. However, at this time much of the indifferent gonad rests on the walls of the subcardinal vein, from which small branches penetrate the mesenchyme under-

lying the germinal epithelium. The gonad begins at the anterior pole of the mesonephros and extends posteriorly for almost two thirds of its length.

An epithelium, three to five cell layers in thickness, extends for some distance over the root of the mesentery and on to the free surface of each genital anlagen. This germinal epithelium is composed of tall columnar cells supported by a distinct basement membrane. The germinal epithelial cells located near the basement membrane have elongated nuclei; those on the free surface possess round nuclei. All nuclei stain deeply. The germinal epithelium over the left gonad measures from 25 to 30 μ, as compared to from 10 to 15 μ for that covering the right gonad.

The gonadal mesenchyme or stroma, which lies beneath the germinal epithelium, is increasing rapidly. This embryonic mesenchyme is continuous with the mesenchyme of the mesonephros and the root of the mesentery. Their nuclei stain faintly, and their cytoplasm is drawn into processes which anastomose with the processes of neighboring cells. The stroma is more loosely arranged near its attachment to the subcardinal vein and mesonephros and is dense next to the germinal epithelial basement membrane.

The stroma, which composes a greater part of the gonads, is more voluminous in the left than in the right gonad. Occasionally, a narrow cord extends obliquely toward the medial surface of the mesonephros. This cord is characterized by a condensation of mesenchymal tissue and is known as the cord of urogenital union.

The primordial germ cells do not as yet form a continuous tissue. They are still found as isolated cells or in groups of two or three in the germinal epithelium; some may be found in the root of the mesentery. These cells are easily identified by their large nuclei and clear cytoplasm. They measure from 10 to 12 μ in diameter.

At 134 hours of development, a marked change is observed in the gonads. The sexual cords have now appeared. These cords, which will become the seminiferous cords in the male and the medullary cords or cords of the first proliferation in the female, occur as buds or protrusions of cells from the germinal epithelium into the underlying mesenchyme or gonadal stroma. These cords of first proliferation cells push the germinal epithelial membrane ahead of them into the stroma, thus giving the basement membrane an undulating appearance. The basement membrane is still continuous around the germinal epithelium. An occasional primordial germ cell is found in the bud.

The rete or urogenital cords are better developed and connect the sexual cords with certain malpighian corpuscles in the mesenephros.

After 148 hours, the production of sexual cords reaches its peak (fig. 2). Many mitotic figures are seen in the nuclei of the cells in the germinal epithelium. Much of the gonad is composed of cords of cells, which lie close together and are separated from one another by a few stroma cells. Some of the cords are no longer attached to the germinal epithelium. A definite base-

Fig. 1—A transverse section taken through the abdominal cavity of a 120-hour chicken embryo showing a portion of the developing gonads. Observe the differences in size and compare the number of primordial germ cells in the left and right gonads.

a) Left gonad; (b) right gonad; (c) primordial germ cell. Hematoxylin-eosin. x 200.

ment membrane surrounds each cord of sexual cells. The right gonad is 1.5 mm. in length, and 0.80 mm. in diameter; the left is 1.65 mm. in length and 1.00 mm. in diameter. The diameters appear to be increasing and the lengths decreasing. The arrangement of the cords is definite and characteristic in that they are straight and extend from the germinal epithelium toward the mesonephros. The largest number of primordial germ cells is found in the germinal epithelium; many are found in the sexual cords; and finally, an occasional one is found in the stroma between the cords. The rete cords are firmly attached to the malpighian corpuscles.

At 165 hours the formation of cords of first proliferation has ceased, and morphological sexual differences may now be observed. In those individuals which are destined to become males, the germinal epithelium has been reduced to a single layer of flat to cuboidal cells. The germinal epithelium covering the left gonad in the female consists of three to five cell layers (fig. 3). These cells are somewhat taller than those in the male. The cords are still attached to the germinal epithelium in the male but are becoming detached in the female gonad.

As many as eight or ten primordial germ cells may be found in one field in the germinal epithelium covering the left gonad

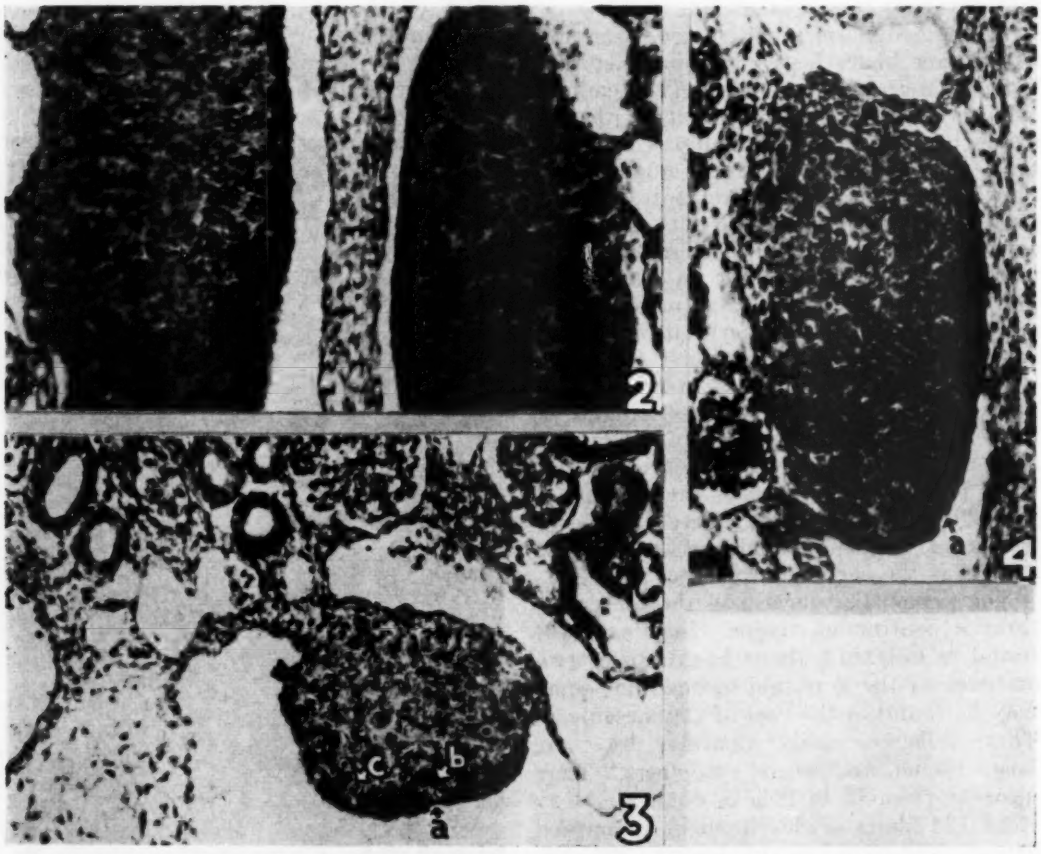

Fig. 2—A frontal section taken through the gonads of a 148-hour chicken embryo. Hematoxylin-eosin. x 200.

Fig. 3—A sagittal section from the left gonad of a 165-hour female chicken embryo. a) Germinal epithelium; (b) shows extent of ingrowth of a primary sex cord from the germinal epithelium; (c) primordial germ cell. Hematoxylin-eosin. x 200.

Fig. 4—A sagittal section taken from the testicle of a 165-hour chicken embryo. Observe the difference in the thickness of the germinal epithelium between figures 3 and 4. a) Germinal epithelium only one or two cells in thickness. Hematoxylin-eosin. x 200.

of the female. Occasionally, a primordial germ cell is found in the germinal epithelium of the male gonad; the majority of these cells have gone into the sexual cords (fig. 4). Marked differences in the relative sizes of the gonads have become apparent.

After 177 hours, the sex of the individual can be easily ascertained, either microscopically or macroscopically. The germinal epithelium covering the left ovary of the female is three to five cell layers in thickness, and cell outlines are difficult to see. The number of cell layers present is based on the cell nuclei which stain distinctly. The basement membrane, which previously was continuous around the growing sexual cords, becomes discontinuous at this time. Mesenchymal cells between the cords, cords of cells derived from the germinal epithelium, and primordial germ cells are found in the tissue beneath the germinal epithelium. That region beneath the germinal epithelium will now be designated as the medullary region. Continued growth of this region is indicated by the presence of many mitotic figures.

The primordial germ cells are more evenly distributed in the germinal epithelium of the left female gonad, but a large number of them remain in the germinal epithelium near the mesentery. The larger cells are 13 μ in diameter, and their cytoplasm is increasing. The nuclei of these cells are 8 μ in diameter. The average cross section contains about six primordial germ cells in the germinal epithelium.

Several differences may be observed between the right and left female gonad. The germinal epithelium of the left gonad is approximately twice as thick as that of the right. There are four to five times as many primordial germ cells in the germinal epithelium of the left gonad as in that of the right. A greater number of primordial germ cells is found in the medullary region of the left gonad than in the right.

DISCUSSION

The origin and migration of the primordial germ cells in the chick is still a subject of considerable controversy. For this reason, and because that particular problem is so involved, the author decided not to include experiments directly concerned with this phase of the investigation. For reviews of the subject, the reader is referred to Heys [17] and Blocker.[3]

Assuming the extragonadal origin of the primordial germ cells, Brode [4] observed that in the chick they persist in the right ovary prior to the third week, after which time they gradually disappear. Benoit,[2] and Domm [9] found that if the left ovary is removed from the chick before the time of the primordial germ cell disappearance, the right rudimentary gonad would transform into a testis containing male sex cells. On the other hand, if the left ovary is removed after the disappearance of the primordial germ cells from the right rudimentary gonad, no sex cells differentiate in the testis. These experiments are the most conclusive evidence that the primordial germ cells are the sole source of the definitive sex cells.

Von Berenberg-Gossler [36] believed that the primordial germ cells are nongerminal in character. He regarded them as entodermal wandering cells which later transform into mesodermal cells; since these cells are similar to other mesodermal cells, they may give rise to sex cells. This view is not widely accepted.

The observations made in this investigation on the origin and migration of the primordial germ cells agree fairly well with those made by Dantschakoff,[5] and Swift.[32] According to Dantschakoff, the primordial germ cells are distributed in the embryo and vascular area until the twenty-second somite stage, at which time they have entirely disappeared. Swift found all the germ cells together with the blood elements in the blood vessels up to the twenty-first somite stage; after this time they gradually disappear and are absent after the twenty-fifth somite stage.

In the 96-hour embryo, the single layer of epithelium covering the gonad is called the germinal epithelium; it was first described in the chick by Waldeyer.[38] At 96 hours, the left gonad is larger than the right. Swift [33] stated the "left gonad is more massive than the right" in the 90-hour embryo. In this connection, Firket [11] mentioned the unequal distribution of the primordial germ cells in the gonads of a 4¾-day embryo. He counted approximately 750 gonocytes in the left, and 150 in the right gonad. Swift [33] counted about twice as many in the left as in the right, in a 4-day embryo, while in a second embryo they were five times more numerous in the left gonad. Dantschakoff [6] stated that at

the twenty-fourth to twenty-sixth somite stage, the left side contains rarely less than twice, and generally three to five times as many as the right side.

The observations made in this investigation do not conform with the account of Dantschakoff.[6] The germ cells in the gonads investigated are nearly evenly distributed up to the end of the third day of incubation. This observation agrees with that of Witschi.[40] During the fourth day of development, this investigation revealed that the left gonads contain approximately 70 per cent, while the right contain 30 per cent, of the total germ cells counted in the gonads of 4 embryos (table 1). At this stage, the mesentery forms between the gonads by the inward movement of the splanchnopleures which fuse. These splanchnopleures contain germ cells, and this accounts for the rather sudden unequal distribution of the germ cells. According to Witschi,[40] immediately after mesenteric formation there is a "passage of more than half of the germ cells from the right to the left side." Swift[33] expressed the opinion that the "unequal distribution of the germ cells in favor of the left side may account for the fact that the gonad of that side is, from its first appearance, the more massive." The unequal distribution of germ cells between the two gonads is rather definite proof that they migrate and are not distributed by tissue displacement as Von Berenberg-Gossler[36] advocated.

By 120 hours, there is a demonstrable difference in the thickness of the germinal epithelium between the left and right gonad (fig. 1). The germinal epithelium covering the left gonad is approximately twice as thick as that of the right. There seems to be a direct proportion between the number of germ cells present in either gonad and the thickness of the germinal epithelium. This observation lends support to Swift's statement above, relative to the differences in size between the two gonads. In this connection, it is also interesting to note that the stroma of the left gonad is approximately twice as thick as that of the right.

Occasionally, at the 120-hour period, a narrow cord is found to extend obliquely toward the medial surface of the mesonephros. This cord of urogenital union is characterized by a condensation of mesenchymal tissue. According to Firket,[11] there are 16 of these cords and they, together with the tissue under the germinal epithelium, make up the urogenital connections or "organ of Mihalkowics" as described by Sainmont.[29] There are differences of opinion with regard to the origin of these cords of urogenital union. The findings in this investigation agree with those of Firket,[11] and Swift,[33] who stated that they arise from mesenchyme. However, Sainmont[29] believed that in the cat these cords arise from the epithelium of Bowman's capsule. Allen,[1] working with reptiles, described them as derived through invaginations of the germinal epithelium. From the above observations, it is noted that the cells making up the cords of urogenital union have all the characteristics of mesenchymal cells, and that they blend with other mesenchymal cells under the germinal epithelium. The cells do not resemble the elongated prismatic cells of the germinal epithelium, nor the flat simple squamous cells of Bowman's capsule. The cords were not counted because some of them were very indefinite.

A marked change occurs in the gonads between 134 and 165 hours (fig. 2). The sexual cords of first proliferation are best seen at this period. Swift[33] observed the period of first proliferation of the sexual cords between 132 and 156 hours. At 165 hours of development, the formation of cords of first proliferation has ceased. According to Swift,[33] these sexual cords cease forming abruptly at 156 hours. The sexual cords are destined to form the seminiferous tubules in the male and the medullary cords in the ovary.

At 165 hours, morphological sexual differences appear in the gonads. Prior to this time, the gonads have shown no structural sex differences. The indifferent period extends from the time of origin of the gonad as a genital ridge, to the time of sex differentiation which, in the material studied, is approximately 165 hours. Swift[33] observed that at about 156 hours of development, the sex of the individual could be definitely determined.

Several criteria are helpful in the determination of sex at 165 hours. These have already been noted by Swift[33] as differences in relative size of the right and left gonad, thickness of the germinal epithe-

lium, size of the cells in the germinal epithelium, and the number and size of the primordial germ cells.

From the time of the indifferent stage of development, the left gonad has been larger than the right. However, at 165 hours the difference in size between the left and right female gonads is more striking. In the male, this difference in relative size between the two gonads is not marked, but the left gonad is slightly larger than the right. The difference in relative size between the gonads in the female is produced by the rapid growth of the cords of first proliferation, and the constant increase in thickness of the germinal epithelium covering the left gonad.

The germinal epithelium of the left gonad of the female is much thicker than the germinal epithelium covering the left gonad of the male at 165 hours (fig. 3, 4). The thin, germinal epithelium covering the right female gonad is similar to that covering the right and left gonads of the male. This difference in thickness of the germinal epithelium is the result of several factors. As previously pointed out, there seems to be a direct correlation between the number of primordial germ cells present in the gonad and the thickness of the germinal epithelium. Thus, as the number of primordial germ cells increases, so also the thickness of the epithelium increases. Another factor contributing to the thickness of the germinal epithelium is the size of the cells. The cells are taller in the epithelium of the left gonad of the female than in the epithelium of the right gonad and that covering the testicles.

At 165 hours, primordial germ cells are numerous in the left gonad of the female, and they are larger than those in the male. Only on occasional primordial germ cell is present in the germinal epithelium of the male gonad. Apparently all these cells have migrated into the sexual cords.

Summary

The morphogenesis of the indifferent gonads of 420 developing chicken embryos was studied by means of serial, sagittal, transverse, and frontal sections taken at approximately twelve-hour intervals from the twelfth hour of incubation through 165 hours of development.

From 90 to 96 hours, a single layer of germinal epithelium covers the embryonic gonad. At this time, the left gonad is larger than the right.

At 96 hours the left gonad contains approximately 70 per cent, while the right contains 30 per cent, of the total germ cells counted in the gonads of 4 embryos.

The sexual cords of first proliferation become well developed between 134 and 165 hours of incubation.

At 165 hours, the morphological sexual differences become apparent in the gonads.

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Transmission of Experimental *Vibrio Fetus* Infection Among Guinea Pigs and Hamsters

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THERE IS evidence that bulls may spread *Vibrio fetus* infection by natural service[6,7] and that such an infection can be induced by artificially inseminating cows with *V. fetus*-infected semen.[1,3]

The following experiment was conducted to determine if infection with *V. fetus* can be transmitted by means of coitus among guinea pigs and among hamsters.

MATERIALS AND METHODS

Virgin guinea pigs, approximately 7 months of age, and virgin hamsters, about 3 months old, were used in these studies. The male guinea pigs were approximately 6 to 12 months old and had been used as breeding animals, while the male hamsters were 3 to 4 months old.

Vibrio fetus, strain K, which was isolated from a flock of Wisconsin sheep, was used in this study. The preparation of Vibrio cultures and the standardization of inoculum density, as well as the technique for isolation of the organisms from the infected tissues of female guinea pigs, was previously described.[4] Antigens were prepared from the Vibrio cultures isolated and the agglutination test[2] was conducted using high-titer *V. fetus*, strain K, rabbit antiserum to verify the identity of the organisms.

For the isolation of vibrios from hamster uteri, small sections (3 to 5 mm.) were placed in tubes of Albimi Brucella semisolid broth.[4] Testes of guinea pigs and hamsters were prepared by grinding these tissues in sterile tissue grinders containing 2 ml. of a sterile solution (0.85) of sodium chloride. One-half milliliter of this fluid was placed in each culture tube of the semisolid medium.[4]

Experiment 1.—An experimental and a control group of guinea pigs were used. Each group consisted of 10 males and 20 females. Two females were placed in each box of the experimental group. One of these was given a vaginal inoculation of 1 ml. of the *V. fetus*, strain K, saline suspension by means of a calibrated capillary pipette on five alternate days. A male was added to each of the ten boxes on the day after the third inoculation.

The animals in the control group were treated in the same manner with the exception that sterile Albimi Brucella semisolid medium was used instead of the Vibrio suspension *per vaginam*.

Blood serum was collected from animals of both experimental and control groups one week before the experiment started and again at one and two months after the last inoculation. The blood serum tube-agglutination test method[2] was used to determine the presence of *V. fetus*, strain K, agglutinins. Cultures and gram-stained smears were made from uterine fluid and fetal stomach contents of animals from both groups at the time of abortion, gestation, or death. Animals which appeared sick were destroyed. All remaining animals were destroyed and tissues cultured for *V. fetus* three months after the last inoculation. Examinations were made daily for one week following the last inoculation, and weekly thereafter, for the presence of any prepucial and vaginal discharges. Gram-stained smears were made of the discharge when present.

Experiment 2.—Ten male hamsters were inoculated intraperitoneally with 0.3 ml. of a saline suspension of *V. fetus*, strain K, as previously described, on days 1, 3, and 5. After the last inoculation, each male was placed in a box with 2 noninfected females. Animals from four boxes were destroyed seven days after the male was placed with the females. Those from three other boxes were killed twelve days after contact and those from the remaining three boxes at twenty-one days. Bacteriological examinations were made from the uterus or testes of each animal.

Two boxes containing 2 female hamsters each were used as controls. An uninoculated male was placed in each box at the time the inoculated males were added in the experimental group.

Blood serum-agglutination tests (*V. fetus*, strain K antigen) were conducted on the serums of all animals at the time they were killed. Organs which gave evidences of gross pathological changes were prepared for histopathological study.[5]

RESULTS

Experiment 1.—Of the 10 female guinea pigs inoculated with *V. fetus*, 2 aborted at thirty-two and thirty-eight days, respectively, after the last inoculation. Two others became sick and were destroyed at forty-three and forty-eight days, respectively. The Vibrio organism was recovered in pure culture from the uteri of all 4 and from the stomach contents of 1 or more of

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the fetuses of each. A vaginal discharge was observed between the third and eighth days after inoculation in the 2 which later

Fig. 1—Testes of hamster destroyed twenty-one days following intraperitoneal inoculation with Vibrio fetus showing thrombus formation and engorged blood vessels. x 75.

aborted. Gram-stained vaginal smears from both guinea pigs contained vibrios. The other 2 inoculated females died at fifty and fifty-four days after the last inoculation. These were pregnant, but the organism was not demonstrable by bacteriological culture. The remaining 4 were negative for *V. fetus* at the time of the last examination. In one instance, parturition occurred at sixty-eight days after the last inoculation. Another was pregnant when she was accidentally killed during the bleeding operation, one month after the last exposure. The remaining 2 were not pregnant when destroyed ninety-one days after the last inoculation.

Four of the 10 uninoculated females which were placed with exposed animals were destroyed at the same times (32, 38, 43, and 48 days) as their exposed partners and all were pregnant. Two of these were bacteriologically negative. *Vibrio fetus* was isolated in pure culture from the uterus and gall bladder of the third animal at forty-three days and from the stomachs of 2 fetuses of the fourth animal at forty-eight days. Four other uninoculated guinea pigs had normal parturitions and were negative. The remaining 2 females were negative. Of these, 1 was destroyed and was not pregnant; the other was pregnant when accidentally killed at the time of bleeding.

Four of the 10 males placed with the inoculated females were destroyed at the same time (32, 38, 43, and 48 days) as their exposed partners. Five others were destroyed later, and 1 died from undeter-

TABLE 1—Results of Experiment 1, Transmission of Vibriosis Among Guinea Pigs

Group	Subgroup	No. of animals	Termination *	Testes-Uterine-Fetal bacteriological examinations	Status
Experimental	Inoculated females**	10	1 accidentally killed at 31 days	Negative	Pregnant
			2 aborted at 32 and 38 days	*Vibrio fetus* in pure culture from both	Pregnant
			2 destroyed at 43 and 48 days	*Vibrio fetus* in pure culture from both	Pregnant
			2 died at 50 and 54 days	Negative	Pregnant
			1 gestated at 68 days	Negative	Pregnant
			2 destroyed at 91 days	Negative	Nonpregnant
	Uninoculated females	10	1 accidentally killed at 31 days	Negative	Pregnant
			2 destroyed at 32 and 38 days	Negative	Pregnant
			2 destroyed at 43 and 48 days	*Vibrio fetus* in pure culture from uterus and gall bladder of 1 and from 2 fetuses of the other	Pregnant
			4 gestated at 69 and 75 days	Negative	Pregnant
			1 destroyed at 91 days	Negative	Nonpregnant
	Uninoculated males	10	9 destroyed at 32 and 91 days	All negative	
			1 died at 69 days	Negative, inflamed testes	
Control	Females†	20	1 accidentally killed at 61 days	Negative	Nonpregnant
			12 gestated at 66 to 79 days	Negative	1 Nonpregnant
			7 destroyed at 91 days	Negative	6 Pregnant
	Males	10	10 destroyed at 91 days	Negative	

* Time after last bacterial inoculation; ** inoculated females received *V. fetus* saline suspension on five alternate days *per vaginam*; † 10 control females received sterile Albimi Brucella semisolid broth on five alternate days *per vaginam*.

mined causes. All were bacteriologically negative. The testes of the animal which died were twice their normal size and a hemorrhagic inflammatory reaction was evident.

All 20 female and 10 male guinea pigs of the control group were negative for *V. fetus* at the time of parturition or destruction. One female of the control group was not pregnant.

The blood serums of all animals one week prior to the start of the experiment were negative for *V. fetus*, strain K, agglutinins. Six females which were bacteriologically positive gave *V. fetus* agglutinin titers of a partial reaction at 1:10 to a complete reaction at 1:40 at the time they were destroyed. The blood serums of all other animals were negative at the times they were examined.

The results of this experiment are summarized in table 1.

Experiment 2.—Vibrio fetus was recovered in pure culture from the testes of 7 male hamsters at seven, twelve, or twenty-one days after their last inoculation. Tissues from the other 3 males were negative. *Vibrio fetus* was recovered from the nongravid uteri of 4 females destroyed at seven, twelve, or twenty-one days after they were placed in contact with males inoculated with *V. fetus*. Three of these 4 infected females were bred by males from which vibrios were isolated at the time of destruction, while 1 cohabited with a male which was found to be negative for vibrios

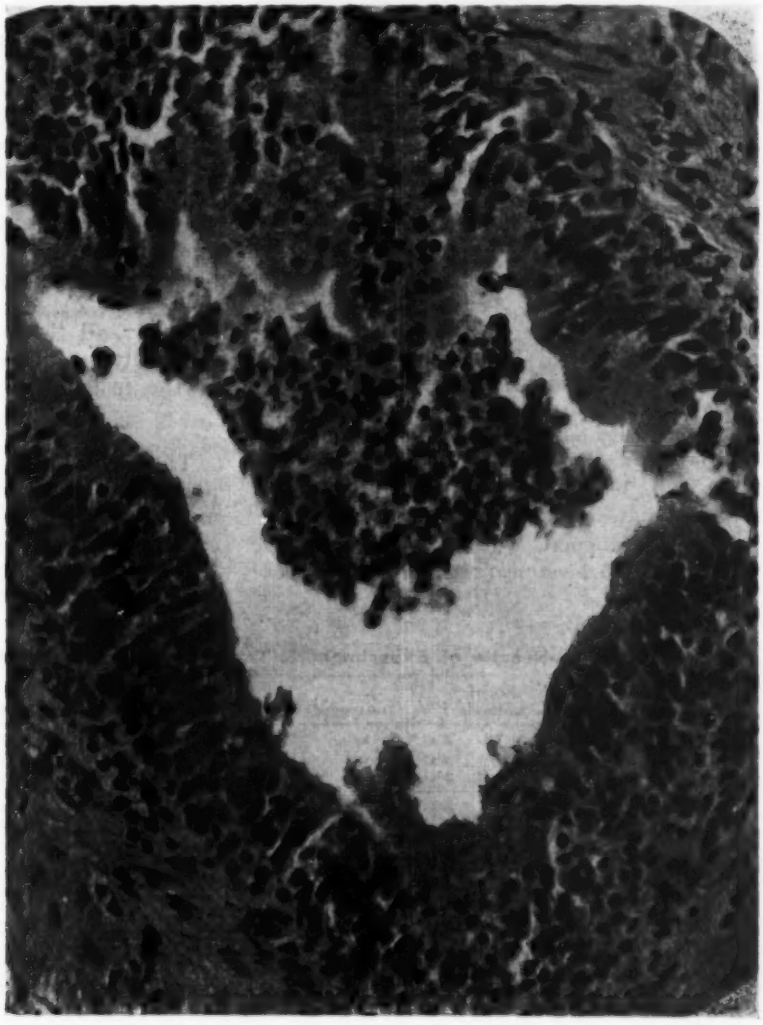

Fig. 2 — Inflammatory cells in the lumen of a duct of a hamster destroyed twelve days following intraperitoneal inoculation with Vibrio fetus. x 300.

when killed. The remaining 16 females of the experimental group were bacteriologically negative for *V. fetus*. One of these

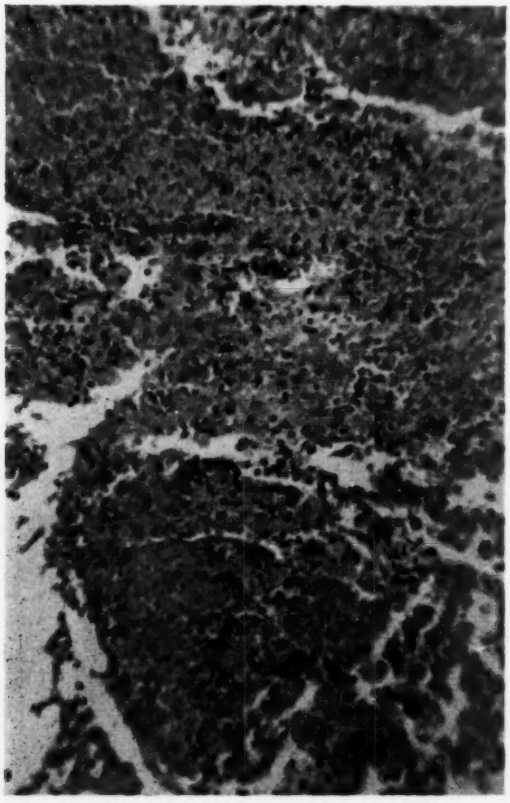

Fig. 3—Testes of hamster destroyed twelve days following intraperitoneal inoculation with Vibrio fetus, showing necrosis and inflammatory cell reaction. x 75.

produced normal young at twenty-one days, while 6 became pregnant and 9 were not pregnant.

Vibrio fetus was not isolated from hamsters of the control groups. The females produced young at nineteen days. The 2 males were destroyed at twenty-one days after being placed in contact with the females.

The blood serum of all inoculated males contained agglutinins for *V. fetus*, strain K, at titers of 1:640 to 1:1,280. The titers of the 4 infected females were 1:10 to 1:40. The serums of the other experimental and control animals were negative.

The summarized results of this experiment are presented in table 2.

Pathological Alterations of the Testes.—The testes of the 7 infected male hamsters were small and firm. When removed and viewed from the exterior, it was possible to detect extensive fibrosis, blood vessel engorgement, hemorrhage, and necrosis in the subcapsular region. These features were even more conspicuous when the tissues were cut and, in addition, pockets containing pus of a caseous nature were prominent. The architecture of the organ was destroyed, partly due to the extensive fibrosis. Thrombosis was evident (fig. 1). The stroma and the few remnants of the seminiferous tubules were infiltrated with polymorphonuclear leukocytes as were the ducts (fig. 2). Necrosis which involved essentially all the seminiferous tubules was especially prominent in the central parts of the testes (fig. 3).

DISCUSSION

These studies demonstrate that guinea pigs can be infected with *V. fetus* by means of the intravaginal route. The conception rate does not seem to be markedly reduced by such an infection but some infected, pregnant animals aborted or died.

TABLE 2—Results of Experiment 2, Transmission of Vibriosis Among Hamsters

Group	Sex	No. of animals	Destroyed*	Testes-Uterine bacteriological examination	Status
Experimental	Inoculated males	10	4 at 7 days	*Vibrio fetus* in pure culture from 3	
			3 at 12 days	*Vibrio fetus* in pure culture from 2	
			3 at 21 days	*Vibrio fetus* in pure culture from 2	
	Females	20	4 at 7 days	*Vibrio fetus* in pure culture from 1**	All nonpregnant
			6 at 12 days	*Vibrio fetus* in pure culture from 2†	All nonpregnant
			3 at 21 days	*Vibrio fetus* in pure culture from 1**	All nonpregnant
			6 at 7, 12 and 21 days	All negative	
			1 gestated at 21 days	Negative	All pregnant
Control	Males	2	2 at 21 days	Negative	
	Females	4	4 gestated at 19 days	Negative	

* Time after last *V. fetus* intraperitoneal inoculation of the males in experimental group; ** cohabitation with bacteriologically positive male; † 1 female with bacteriologically positive male and 1 with negative male.

Recovery of *V. fetus* from the uterus and gall bladder of 1 uninoculated guinea pig, and from the stomach contents of the fetus of another indicated a natural spread of infection by coitus. Since *V. fetus* was not cultured from the testes of any of the males in this group, it is probable that transmission was merely mechanical and did not involve active infection and establishment of the organism in the male reproductive tract for any period of time.

One may conclude from experiment 2 that an active infection can be produced in male hamsters by means of intraperitoneal inoculation. Also, since *V. fetus* was recovered from the uteri of 4 noninfected females, it appears that the infected males may transmit infection by coitus. While all of the control hamster females experienced normal parturitions, 65 per cent of the females experimentally infected with *V. fetus* failed to conceive. It is a matter of speculation whether this infection interfered with the actual fertilization, produced embryonic death, or interfered with the formation of the sperms. The latter is a distinct possibility, since testicular damage was of such magnitude that the males were probably rendered sterile.

An important finding in both experiments was a lack of or low level of agglutinins for *V. fetus* in the blood serum of contact animals. This may indicate that an infection by the vaginal route does not greatly stimulate a reticuloendothelial response. This might explain the absence of or the low agglutinin titers in the blood serum of some infected cows. This further substantiates the possibility that intravaginal infection with *V. fetus* is of a local character involving chiefly uterine tissue changes without sufficient general body reaction to form serum antibodies.

SUMMARY

Nonpregnant guinea pigs which were infected intravaginally with *Vibrio fetus* became pregnant and then some aborted. When such infected females were kept with noninfected females and males and breeding was allowed, the infection was transmitted to a few uninoculated females. *Vibrio fetus* agglutinin titers of all infected females ranged from 1:10 to 1:40. The males did not develop titers.

When 10 male hamsters were inoculated intraperitoneally with *V. fetus* the organism was recovered at seven, twelve, and twenty-one days from the testes of 7. They had transmitted infection by coitus to noninfected females.

Vibrio fetus blood serum-agglutinin titers in all infected female hamsters ranged from 1:10 to 1:40 while titers of 1:640 to 1:1,280 were observed in the males.

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Rapid Diagnosis of Newcastle Disease Using Lung Extract

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THE ABILITY of Newcastle disease virus (NDV) to agglutinate red blood cells, first observed by Burnet [1] in 1942, is widely used as an aid in the diagnosis of the disease. In order to demonstrate the hemagglutinating activity (HA) of the virus, it must first be cultivated in embryonating eggs of which infected allantoic fluids are employed in the test. The use of antibiotics to eliminate bacterial contaminants has simplified virus isolation, but the harvested allantoic fluids must still be cultured for bacteria and known ND immune serums employed either in hemagglutination-inhibition or serum-neutralization tests to ascertain the specificity of the embryonic deaths and of the hemagglutination. The isolation procedure preliminary to demonstration of HA may take two to six days, a period that may be too long to permit initiation of prompt control measures in an outbreak of ND. Demonstration of circulating antibodies which inhibit hemagglutination is a rapid technique but must await their development by the infected fowl, generally seven or more days after infection has occurred. A method for the rapid determination of the presence of Newcastle disease virus when an outbreak is still in its first stages would have considerable practical value.

Sinha, Hanson, and Brandly [2] found that 6- to 7-week-old chickens exposed to aërosols of six strains of NDV and destroyed after ninety-six hours had a titer of the virus as high as 10^7 $l.d._{50}$ in the lung tissue. Similar findings have been reported by others [2, 3] using different strains of NDV and parenteral routes of inoculation. Since hemagglutinative activity is usually demonstrable in suspensions of virus with an infective titer of 10^6 $l.d._{50}$ per milliliter, the possibility of determining the presence of Newcastle disease using an extract of lung tissue and the HA technique was studied.

METHODS AND RESULTS

Chickens, 8 weeks old, were exposed by aërosol, in a manner that simulated field exposure, to two strains of Newcastle disease, GB and Roakin, 12 birds receiving each strain. All birds that developed signs of ND were killed when the signs became evident. Those not developing overt disease were destroyed on the sixth day following exposure. Suspensions of lung tissue prepared in Ten-Broeck glass grinders were adjusted to pH 7, and elution of the virus was allowed to proceed at 37 C. or 50 C. Suspensions which had been eluted in a water bath at 50 C. for one hour gave consistently higher virus titers than those eluted at lower temperatures (table 1).

A method which avoided grinding was attempted. Lung fluid was extracted by means of a garlic press. Extracts of the fresh lung contained tissue debris that interfered with the reading of the test. When the lungs were frozen and then pressed in the frozen state the hemagglutination tests could be interpreted more readily and the titers were higher.

Subsequently, the test was used as a diagnostic aid at a large poultry farm where a mild form of ND was enzoötic. The lungs of suspected birds which had conjunctivitis or were coughing and sneezing were frozen at −17 C. Five drops of fluid were expressed directly into 0.8 ml. of saline solution and the material eluted in a water bath for one hour at 50 C. From the initial group, isolations of NDV were attempted with specimens showing an HA titer. Virus was not obtained from lungs whose extracts had a titer of 1: 10 in the three instances in which it was tried, but all of the four lungs which had HA titers of 1: 40 and 1: 80 yielded virus.

DISCUSSION

The experiments described indicate that lung tissue extracts of chickens affected with ND may possess significant HA activity and the demonstration of this activity is a rapid procedure for the diagnosis of Newcastle disease. It appears that the most critical factor in the procedure is the freezing of the lung tissue in order that tissue fluid may be expressed while the lung is in the frozen state.

Further examination of this diagnostic procedure is planned. The absence of HA in the lungs of chickens would not eliminate a diagnosis of NDV as the virus that sometimes may be found in such individuals, particularly those having a mild form of the disease. Nevertheless, positive findings in flocks suffering from a respiratory infection is significant and warrants field

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TABLE 1—Effect of Treatment of Tissue on Hemagglutination Titer

Treatment				Hemagglutination titer						Result
Initial freezing	Elution temperature	Group	Disease	0	10	20	40	80	160	No. HA/ No. deceased
none	37	A	overt	8	0	0	1	0	0	1/12
			subclinical	3	0	0	0	0	0	
none	37	B	subclinical	12	0	0	0	0	0	0/12
none	50	A	overt	0	2	5	0	1	1	11/12
			subclinical	1	2	0	0	0	0	
none	50	B	subclinical	3	6	2	0	0	0	8/12
yes	50	C	overt	0	0	1	1	2	2	6/6

Group A—12 chickens exposed to an aërosol of GB strain of Newcastle disease virus; 9 developed respiratory and nervous signs in five days; 3 failed to develop overt disease.

Group B—12 chickens exposed to an aërosol of Roakin strain NDV; all 12 failed to develop overt disease.

Group C—6 chickens exposed to an aërosol of GB strain NDV; all 6 developed respiratory and nervous signs.

trial. Newcastle disease antiserum should be used in the extract-hemagglutination test to determine the specificity of the hemagglutination. Availability of fowl plague antiserums would permit rapid diagnosis in instances of high flock mortality in which the hemagglutination was not inhibited by ND antiserums.

SUMMARY

A procedure for the rapid diagnosis of Newcastle disease is described. Extracts of frozen lungs were taken from chickens suffering from the acute disease and tested for their ability to agglutinate erythrocytes. The results of the test were obtained within two hours after receiving the suspected tissue and the inhibition of the agglutination permitted differentiation from other respiratory infections. The limitations of the method are discussed.

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Partially Purified Dried Epsilon Toxin and Toxoid as Reagents for the Standardization of *Clostridium Perfringens* Type D Bacterin

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Enterotoxemia ("pulpy kidney disease," "overeating disease") of sheep and goats has been described in Australia, England, the United States, and other countries of the world,[1] and it has been considered to be due to the elaboration of *epsilon* toxin in the intestine by *Clostrodium perfringens* type D.[1,2] This disease has been successfully controlled by immunization with formalin-killed whole culture bacterin which is widely employed in England and Australia as well as in this country.[1-6] The major active principle in the formalized whole culture is the *epsilon* toxin, which is converted to the immunologically active toxoid by the addition of formalin.[3] Most bacterins in practical use contain potassium alum as an adjuvant.[6]

Methods for the standardization of *Clostridium perfringens* type D bacterins have been reported by various workers.[2,5,7-9] In the absence of a suitable active protection test, the antitoxin combining power of toxoids has been suggested as an index of antigenicity.[5] The combining power test has been shown to be exceedingly sensitive to relatively minor changes in conditions[9] and, if reproducible results are to be expected among different laboratories, the test must be defined within comparatively narrow limits. It is the purpose of this report to describe two reagents, an activated dried toxin and a dried toxoid, that are both stable and suitable for the standardization of the combining power test in the titration of *Cl. perfringens* type D bacterins.

Materials and Methods

Definitions.—The definitions adopted are those previously reported[9] and, in most instances, they conform closely to those of Hejl[7] and de Mello and Kiser.[10]

1) One toxin unit (m.l.d.) is the minimal amount of toxin, diluted in beef infusion broth, that will kill 5 out of 5 mice within forty-eight hours when given intravenously in a final volume of 0.3 ml. to white Swiss female mice weighing 18 to 24 Gm.*

2) One antitoxin unit is the minimal amount of antitoxin that will neutralize exactly 15 toxin units of fresh activated toxin when the mixture is incubated for one hour at 25 C.

3) One combining unit of toxoid is the minimal amount of toxoid that, when incubated for one hour at 25 C. in the presence of 1 unit of antitoxin, will neutralize it to such an extent that on the addition of 2 units of toxin and reincubation for one hour at 25 C., 0.3 ml. of the mixture injected intravenously will kill at least 2 out of 3 mice, each weighing 18 to 22 Gm.

Preparation of Toxin.—The toxin used was a partially purified dried toxin prepared by a modification of the method of Turner and Rodwell[11] as follows: A culture of *Cl. perfringens* type D, isolated from a case of enterotoxemia, was grown for six days at 37 C. in a beef heart infusion broth containing dextrose. The six-day incubation insured maximal yield of ϵ prototoxin but almost complete deterioration of the α and θ toxins. The broth culture was freed from its cells by filtration and the material was brought to 37 C. The prototoxin was activated by proteolytic conversion into ϵ toxin by the addition of 0.125 per cent trypsin (Wilson † 1:240) and incubation at 37 C. for forty-five minutes. The material was held at room temperature for two hours and saturated with solid ammonium sulfate (technical grade). The mixture was allowed to stand at 5 C. overnight and a brown precipitate which rose to the top of the mixture was collected by filtration through Whatman No. 5 filter paper and washed with a saturated solution of ammonium sulfate. The toxin was dissolved in 1/6 volume of M/15 phosphate buffer at pH 7.1, shell frozen in an alcohol and dry ice bath, and dried by lyophilization. The dried material was stored at room temperature in a desiccator containing "drierite."

Preparation of Toxoid.—The toxoid used was prepared in a manner similar to the toxin as follows: A culture of *Cl. perfringens* type D was grown as described above. The toxin was detoxified by incubation at 35 C. for fifteen days in the presence of 0.5 per cent formalin. The culture was shaken daily during the period of detoxification. The detoxified material was stored at 5 C. for one week and then the cells were removed by filtration. The filtrate was saturated with solid ammonium sulfate at 5 C. and held at this temperature overnight. The toxoid, which rose to the top of the mixture, was separated and treated in an identical manner at the toxin. The lyophilized material was stored at room temperature in a desiccator containing drierite as a dehydrating agent.

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* The mice used were the C.F. No. 1 strain obtained from the Carworth Farms, New York.

† Purchased from the Wilson Laboratories, Division of Wilson Co., Chicago, Ill.

Standard Antitoxin. — A commercial *Cl. perfringens* type D antitoxin of equine origin was clarified by filtering through a 10-lb. test Mandler candle. The antitoxin was dispensed in 1-ml. amounts and desiccated by lyophilization. The vials were evacuated and stored at 5 C. The antitoxin was carefully standardized against fresh *Cl. perfringens* type D *epsilon* toxin.

TABLE 1—Stability of Dried Clostridium Perfringens Type D Epsilon Toxin

Toxin (mg.)	6 mo.	15 mo.	21 mo.	28 mo.
0.0017	SSS	DDS	DDDDS	DDDDS
0.0035	DDD	DDD	DDDDD	DDDDD
0.0070	DDD	DDD	DDDDD	DDDDD

Each letter represents one mouse; S = Survived; D = Died within forty-eight hours.

Diluent.—The diluent used throughout was a beef heart infusion containing 0.5 per cent sodium chloride. The pH of the diluent was adjusted to 7.0 with normal sodium hydroxide solution.

Test Procedure.—The tests for toxicity and combining power are those previously described.[9]

RESULTS

Stability of the Dried Toxin.—The toxicity of the partially purified preparation of activated toxin, prepared as described, was determined at various intervals over a period of twenty-eight months by injecting intravenously 0.3 ml. of various concentrations of the toxin into each of 3 to 5 mice. The dilutions were made in beef heart infusion at pH 7.0. The toxin solutions were made by weighing accurately 100 mg. of the dried toxin and then diluting it with 100 ml. of broth. From this stock solution, the rest of the dilutions were made, always transferring amounts not less than 1 ml. so as to avoid errors in pipetting. The toxin dilutions were kept for one hour at room temperature before injection. Table 1 gives the results of typical experiments performed over a period of twenty-eight months.

It can be seen that it required 0.0035 mg. of the toxin to kill all of the inoculated mice at each interval. It is unfortunate that the initial toxicity of the preparation was not determined using broth as a diluent. The initial toxicity determined using saline diluent was 0.0156 mg. This has been shown previously [9,10] to yield a toxicity of approximately one fourth that obtained by using broth, which is about the difference obtained here. Thus, the toxicity of the dried preparation may be assumed to be stable up to at least twenty-eight months, the time of the last testing of this material.

Stability of the Dried Toxoid.—The stability of the partially purified preparation of toxoid, prepared as described, was determined by measuring the combining power of the toxoid at various intervals over a period of eleven months. The combining power was determined as follows: Ten units of antitoxin contained in 1 ml. were put into a series of tubes to which 1 ml. of various dilutions of toxoid were added. The toxoid solutions were made by weighing accurately the dried toxoid and then diluting it with beef heart infusion using the same precautions previously described for the toxin. The mixture of toxoid and antitoxin was incubated for one hour at room temperature and then the indicator toxin contained in 1 ml. was added to each tube. The indicator dose used throughout the study was 0.0070 mg. per mouse or the equivalent of 2+ m.l.d. of the standard dried toxin described above. The toxin dilution was prepared in beef heart infusion in a manner similar to the toxoid. After the addition of the indicator toxin, the mixture was further incubated for one hour at room temperature and 0.3 ml. injected intravenously into each of 5 mice. If sufficient antitoxin has combined with the toxoid, the mice will die from the effects of the indicator toxin, while if the combination is incomplete, enough free antitoxin is left to neutralize the indicator toxin and the mice will survive. Table 2 gives the results of typical experiments performed over a period of eleven months.

TABLE 2—Stability of Dried Clostridium Perfringen Type D Toxoid

Toxoid (mg.)	Results			
	0 mo.	4 mo.	8 mo.	11 mo.
0.0340	SSSSS	SSSSS	SSSSS	SSSSS
0.0507	SSSSS	SSSSS	SSSSS	SSSSS
0.0723	DDDDD	DDDDD	DDDDS	DDDDD
0.3400	DDDDD	DDDDD	DDDDD	DDDDD
	Controls			
Toxin-Antitoxin* Toxoid**	SSSSS SSSSS	SSSSS	SSSSS	SSSSS

* To 1 unit of antitoxin contained in 1 ml. was added 1 ml. of indicator toxin solution and 1 ml. of diluent. The mixture was incubated for one hour at room temperature and then 0.3 ml. was injected into each mouse.

** Each mouse was injected intravenously with 0.3400 mg. of toxoid contained in 0.3 ml. of diluent.

It can be seen that the amount of toxoid necessary to neutralize 1 unit of antitoxin to such an extent that at least 4 out of 5 mice are killed by the indicator toxin does not vary over the period tested. It may be concluded from these results that the partially purified dried toxoid is stable at room temperature for a least eleven months which was the latest date of testing.

DISCUSSION

In a previous publication,[9] it was shown that the combining power test for the standardization of *Cl. perfringens* type D bacterin was dependent on many variables which were difficult to control. It was then suggested that "if a reference *Cl. perfringens epsilon* toxoid were made available from a central laboratory together with a pretitrated antitoxin, the titration of an unknown bacterin could be performed satisfactorily regardless of variations in the locally prepared reagents." Although it is a simple matter to preserve standard antitoxin, it has been our experience that liquid bacterin (toxoid) is not stable and gradually loses its combining power for antitoxin. In the present report, however, it has been shown definitely that when toxoid is prepared and desiccated as described it keeps its combining power unaltered for at least eleven months at room temperature and there is reason to believe that it would be stable for a much longer period of time since the toxicity of the toxin which usually is a more unstable property can be preserved in a lyophilized form for at least twenty-eight months. The stability of this preparation of toxoid makes it possible for various laboratories to standardize their bacterin preparations against a standard dried toxoid, thus stabilizing the relative potency of new bacterin preparations. If in addition to a reference toxoid, a reference toxin and antitoxin are used, the actual number of combining power units in a given bacterin can be determined accurately as illustrated by the results reported here.

SUMMARY

1) The preparation of an indicator toxin activated before partial purification and dried by lyophilization has been described. This toxin preparation has been shown to be stable for at least twenty-eight months at room temperature.

2) The preparation of a partially purified dried toxoid, which has been shown to be stable for at least eleven months at room temperature, has been also described.

3) The use of a dried *Clostridium perfringens* type D toxoid as a standard for the standardization of commercial bacterins is suggested.

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The Detection of Residual Hormone in the Meat of Animals Treated with Synthetic Estrogens

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DURING the past decade, numerous investigations have been made of the possible uses of hormones for the more abundant and efficient production of meat, milk, and eggs. The most common application of endocrinology to food production is the implantation of diethylstilbestrol pellets in broiler and frying chickens. This has become a common procedure in commercial poultry production. It has been also demonstrated that diethylstilbestrol, and other estrogens, increase the rate of gain and the efficiency of feed utilization in cattle and sheep (*see* Sykes *et al.*[8] for a complete literature review). However, this technique has not yet been adopted as a routine practice in livestock management since it is not known whether the residual hormone in the meat would make it unsafe for human consumption.

Bird *et al.*[1] demonstrated that in chickens, oral administration of 241.8 mg. of the dimethyl ether of diethylstilbestrol resulted in the recovery of 3.17 mg. of estrogen per 100 Gm. of adipose tissue. However, Greenblatt and Brown [4] were not able to recover estrogen from the fat of a cockerel treated with 30 mg. of diethylstilbestrol subcutaneously and Gowe [3] was able to recover estrogen from the skin, muscle, and liver but not from the adipose tissue of a turkey treated subcutaneously with 48 mg. of diethylstilbestrol.

All of these assays involved the extraction of the tissue with a solvent and injecting the extract into test animals. In view of the work of Teague and Brown [10] who reported that the quantitative recovery of diethylstilbestrol from tissues as determined by colorimetric reactions with antimony pentachloride ranged from 0 to 70 per cent, it is possible that such extractions would not remove all the hormone present in the tissues.

This paper presents a simple procedure for the determination of estrogens in meat from hormone-treated animals. Since these assays were made by feeding the material to ovariectomized mice, it was not necessary to extract the hormone. Thus, no loss could occur from extraction and purification. This method has an additional advantage in that it simulates the manner in which the human being would be exposed to the hormone-treated meat.

MATERIALS AND METHODS

The test animals used for assay purposes were ovariectomized Rockland-Swiss mice. The gonads were removed under ether anesthesia at 2 to 3 months of age. One group of mice were autopsied at weekly intervals for six weeks to determine the relationship of time to uterine atrophy.

A comparison was made between the vaginal smear assay and the assay based on an increase in uterine weight to determine differences in the sensitivity of the two methods. In this determination, diethylstilbestrol was thoroughly mixed with a commercial animal ration which was then fed to the ovariectomized mice for six days. Vaginal smears were taken each day of the six-day feeding period by aspirating a few drops of distilled water in the vagina of the test mice with a small eye dropper. The smear was allowed to dry, fixed with ethyl alcohol, and stained with Giemsa. A positive smear was represented by either nucleated epithelial cells or cornified epithelial cells or a combination of the two with less than 1 per cent of the total number of cells being polymorphonuclear leukocytes. Any increase in the number of leukocytes rendered the smear negative. If the level of hormone in the feed was high enough to elicit a positive vaginal response, the positive smears usually appeared on the fifth or sixth day of feeding. With respect to the uterine weight assay, the mice

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TABLE 1—Uterine Atrophy Following Ovariectomy

Group	Time after ovariectomy (weeks)	No. mice	Mean body weight (Gm.)	Mean uterine weight (mg.)	Body weight (%)
1	0	6	23 (21 to 25)	74.17 ± 11.67	0.322
2	1	5	22 (20 to 27)	22.80 ± 7.89	0.104
3	2	5	23 (21 to 26)	16.56 ± 2.85	0.069
4	3	5	23 (22 to 26)	15.76 ± 0.94	0.066
5	4	5	26 (22 to 28)	16.24 ± 1.66	0.062
6	5	5	23 (20 to 26)	12.28 ± 0.74	0.054
7	6	5	24 (17 to 29)	8.48 ± 0.51	0.037

were killed twenty-four hours after the last feeding. Statistically significant increases in uterine weights were considered to constitute a positive response.

A standard dose-response to diethylstilbestrol was determined by feeding varying amounts of the hormone incorporated in the mouse ration for a ten-day period. In determining the estrogen activity of meat, the mice were fed test samples for a ten-day period and destroyed twenty-four hours after the last feeding. At necropsy the uteri were removed, freed of surrounding tissue, opened lengthwise, and weighed immediately on a torsion balance. The meat used was obtained from steers which had been implanted with pellets of diethylstilbestrol alone, diethylstilbestrol plus pellets of progesterone, or injected with an emulsified, liquid hormonal preparation which contained both dienestrol and progesterone. The implantations or injections were made subcutaneously in the neck region of steers 168 days prior to slaughter. Meat samples used were neck trim which were ground to a hamburger-like fineness and air dried. After drying, the meat sample was weighed and incorporated in a mouse diet which contained 5 parts of meat and 1 part of the usual laboratory mouse mash. The meat-mash mixture was fed to the mice *ad libitum*. Vaginal smears were made on all mice prior to each assay in order to verify completeness of ovariectomy. Statistical analyses were carried out by the method of analysis of variance.[8]

TABLE 2—Vaginal Smear Assay for the Detection of Orally Administered Diethylstilbestrol

Group	No. mice	Diethylstilbestrol μg./Gm. ration	No. Positive	Positive (%)
1	9	0.220	9	100
2	11	0.165	11	100
3	11	0.110	11	100
4	11	0.055	11	100
5	10	0.022	6	60
6	9	0.022	5	55
7	9	0.0165	2	22
8	11	0.011	1	9
9	7	0.0055	0	0

RESULTS

Since it was apparent that the lapse of time between ovariectomy and the use of the animal for assay would influence uterine weight, a preliminary test was conducted to determine the effect of time on uterine atrophy. The results presented in table 1 indicate that uterine atrophy is virtually complete two weeks after removal of the ovaries. Consequently, in assaying the meat for hormonal activity, all the mice that were used had been ovariectomized three to five weeks previously. This lapse of time allowed for uterine regression to be completed.

Data on the comparative sensitivity of the uterine weight and vaginal response of ovariectomized Rockland-Swiss mice to orally administered diethylstilbestrol are presented in tables 2 and 3. If the unit for the vaginal smear technique is based on the presence of a positive reaction in 50 per cent of the mice, then the minimal detectable amount, using the vaginal smear assay, is 0.022 μg. of diethylstilbestrol per gram of ration. The minimal detectable amount, as evidenced by statistically significant differences using the uterine weight assay, is 0.0055 μg. of diethylstilbestrol per gram of ration. These data indicate that under the conditions of this experiment, the uterine weight assay for orally administered diethylstilbestrol is approximately four times more sensitive than the vaginal smear. This difference in the sensitivity of the two methods is in agreement with the work of Rubin *et al.*[5]

In order to establish a means of refer-

TABLE 3—Uterine Weight Assay for the Detection of Orally Administered Diethylstilbestrol

Diethylstilbestrol μg./Gm. ration	No. mice	Mean body weight (Gm.)	Mean uterine weight (mg.)	Body weight (%)
.....	8	24 (21 to 29)	23.57 ± 1.54	0.095
0.0055	8	27 (23 to 29)	40.97 ± 2.76**	0.151**
.....	9	24 (18 to 29)	14.77 ± 1.12	0.06
0.0055	9	33 (30 to 38)	27.95 ± 2.32**	0.08*
.....	10	27 (23 to 33)	9.50 ± 1.30	0.04
0.0055	10	29 (25 to 35)	18.74 ± 2.71**	0.06*
0.0055	9	29 (24 to 40)	17.70 ± 2.16**	0.06**
.....	9	30 (26 to 34)	12.98 ± 1.42	0.04
0.0055	8	31 (27 to 33)	19.10 ± 1.08**	0.06**
.....	10	30 (27 to 33)	15.60 ± 1.89	0.05
0.0033	11	28 (24 to 29)	19.90 ± 1.46	0.07*
0.0044	7	31 (24 to 39)	18.00 ± 1.87	0.06

* $P < 0.05$; ** $P < 0.01$.

TABLE 4—Standard Dose Response to Oral Diethylstilbestrol Based on Uterine Weight Response

Diethylstilbestrol μg./Gm. ration	No. mice	Mean body weight (Gm.)	Mean uterine weight (mg.)	Body weight (%)
.....	7	27 (23 to 33)	16.88 ± 1.89	0.062
0.0055	8	24 (19 to 28)	41.62 ± 6.56	0.166
0.0100	8	26 (21 to 31)	51.42 ± 4.31	0.194
0.0250	8	28 (22 to 31)	104.62 ± 7.87	0.376
0.0500	8	27 (25 to 31)	145.38 ± 9.46	0.534
0.1000	7	27 (24 to 31)	160.37 ± 8.46	0.587

ence for hormonally induced increases in uterine weight, a standard dose-response curve was determined for the orally administered diethylstilbestrol. These results were obtained by feeding diethylstilbestrol-treated mash to the test mice for a ten-day period (table 4).

The results of assaying the meat of hormone-treated steers are presented in table 5. For purposes of statistical analyses, group 1 served as the control for groups 2, 3, and 4; group 5 was the control for groups 6, 7, and 8. With only 1 exception, the mice fed the meat from steers treated with hormones had significantly heavier uterine weights than mice fed meat from nontreated steers. In 3 of the cases, these differences also applied when the uterine weights were expressed as a percentage of body weight. Referring to table 4, it would seem safe to conclude that the amount of residual hormone in beef obtained from estrogen-treated steers does not exceed 0.01 μg. per gram of dried meat.

DISCUSSION

There is a need of quantitative information regarding the estrogenic activity of the meat of normal animals and those which have been treated with estrogenic hormones. This study was designed to measure estrogenic activity in the meat of hormone-treated cattle using the mouse uterine weight method and oral administration of the meat samples. This eliminates the possibility of incomplete extraction and destruction of estrogenic activity during extraction and purification. This method also attempts to simulate the oral exposure which might occur in man. These data indicate that 168 days after the diethylstilbestrol or dienestrol treatment of steers, detectable residual estrogens are present in the meat but in very small amounts.

Since most work on the metabolism of estrogens indicates rapid passage from the organism, it is interesting to speculate on possible explanations for the presence of estrogens in beef 168 days after treatment of the steers. Probably not all of the hormone is present in the fat since West [11] demonstrated that in the case of testosterone the fat contained the highest amount of hormone immediately after intravenous administration but that three hours later there was no hormone present. Szego and Roberts [9] have indicated that estrogen may be protein bound and Bischoff *et al.*[2] reported that muscle cells were freely permeable to estrogen and contained 2.5 times as much estrogen as adipose tissue after in-

TABLE 5—Assay of Beef Muscle for Estrogens 168 Days After Hormonal Treatment

Group	Steer treatment	No. mice	Mean body weight (Gm.)	Mean uterine weight (mg.)	Body weight (%)
1	control	11	25 (22 to 29)	12.25 ± 0.72	0.054
5		12	26 (21 to 30)	15.70 ± 1.44	0.061
2	108 mg. diethylstilbestrol	10	24 (21 to 28)	18.42 ± 1.43**	0.078**
6		9	21 (19 to 25)	14.44 ± 0.65	0.069
3	60 mg. diethylstilbestrol	10	26 (25 to 28)	15.64 ± 1.22*	0.058
7	200 mg. progesterone	9	26 (22 to 30)	31.22 ± 2.39**	0.119**
4	120 mg. dienestrol	10	27 (25 to 29)	19.44 ± 0.90**	0.073**
8	300 mg. progesterone	10	29 (25 to 35)	19.54 ± 0.90*	0.068

* $P < 0.05$; ** $P < 0.01$.

travenous injection of estradiol in the rabbit. A possible explanation for the presence of estrogen 168 days after treatment, is that with high blood titers of estrogen immediately after treatment, there might have been a passage of hormone into the muscle cells where it could have been protein bound. It might then diffuse slowly back into the circulation only when the estrogen concentration in the blood was lower than that in the cells. If the protein-estrogen aggregate is too large to cross the cell membrane there might be no reëntry into the blood. This particular explanation has been made by West *et al.*[12] with respect to the metabolism of testosterone although they do not mention the possibility of the protein binding of androgen.

In view of the knowledge of absorption rates of hormones from pellets, it seems improbable that there would be any pellet residue after 168 days which would liberate hormone into the circulation. Furthermore, in the case of the treatment involving the combination of 120 mg. of dienestrol and 300 mg. of progesterone, the hormones were not in pellet form but in a semisolid base. This would eliminate pellet residues and yet the assay procedures used in this paper detected residual hormone in the meat of steers treated in this manner. A detailed report demonstrating the presence of residual estrogens in beef muscle and liver, lamb muscle and liver, and chicken carcasses is presented elsewhere.[7]

SUMMARY AND CONCLUSIONS

A technique is described for the detection of estrogens in the meat of hormone-treated animals.

Under the conditions of this experiment, the uterine weight assay is four times more sensitive for the detection of orally administered diethylstilbestrol than the vaginal smear method.

Residual hormone activity has been demonstrated in beef secured from steers 168 days after treatment with synthetic estrogens and progesterone. Possible explanations have been offered to account for its presence.

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Electron Microscopic Studies of the Bivins Papilloma Virus

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THE HUMAN papilloma virus for these studies was obtained from Dr. James A. Bivins [1] of Rutgers University. Dr. Bivins isolated this virus from a wart on his hand. The virus was adapted by Dr. Bivins to the membrane of 10-day-old embryonating chicken eggs,[2] and on receipt at this laboratory it had been carried through 12 egg membrane passages. It was carried one additional passage on the membrane of 10-day embryos before initiation of the present study. Melnick *et al.*[3] did electron microscopic studies on the human papilloma virus (but not from papilloma virus that had been adapted to membranes of chicken embryos). He found the virus particles to be spherical, averaging 52 mμ in diameter.

MATERIALS AND METHODS

Lyophilized chorioallantoic membrane of the eleventh egg membrane passage was diluted to a 10 per cent suspension with sterile physiological saline. Twenty 10-day-old chicken embryos were inoculated on the chorioallantoic membrane with this suspension using sterile technique throughout. The eggs were candled twice daily, and upon the fifth day postinoculation the membranes were very congested and thickened. When this was observed embryos were destroyed, and the membranes were recovered from the infected eggs. No attempt was made to determine the mortality rate since all the embryos were destroyed before death. Twenty 10-day-old chicken embryos were inoculated on the chorioallantoic membrane with a 10 per cent suspension of 10-day normal chorioallantoic membranes. On the fifth day postinoculation, the membranes appeared normal and were removed aseptically as above. The pools of infected chorioallantoic membranes and the pools of normal chorioallantoic membranes were ground separately with alundum and diluted to a 20 per cent suspension with physiological saline. The two suspensions were then subjected to five minutes of centrifugation in an angle centrifuge at 1,000 r.p.m. The supernatants were recovered from the specimens and filtered through type ST, size L3 Seitz filters. The filtrates were then subjected to centrifugation for two hours at 44,400 r.p.m. under refrigeration in a Spinco ultracentrifuge. The temperature of the refrigerated outer jacket stayed constant at −8 C. during centrifugation. After the two-hour centrifugation period, the supernatants from the specimens were discarded, and the sediment from each specimen was resuspended in 1 cc. of physiological saline. Several drops of each suspension were placed on several parlodion film supports which had been prepared seventy-two hours previously. After all excess fluid had been removed with small capillary pipettes, the films were dried and shadowed with chromium [4] at arc tangent 2/12 and examined under the RCA electron microscope, type EMU. The balance of the filtered material of the infected specimen was inoculated onto the chorioallantoic membrane of 6, 10-day-old embryonating chicken eggs.

The membranes injected with the filtered infected material showed thickening of the membrane after five days as found when normal chorioallantoic membranes were injected with the original Bivins papilloma virus.

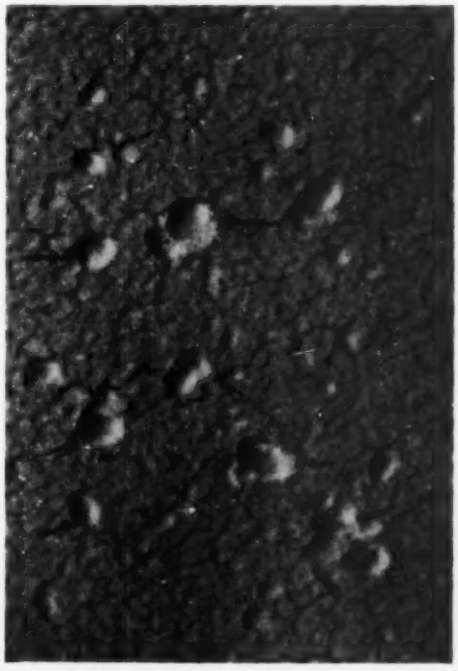

Fig. 1—Bivin's human papilloma virus. Shadowed with chromium at arc tangent 2/12. x 72,000.

From the Virus Laboratory, Livestock Sanitary Service, University of Maryland, College Park; professor of virus diseases (Reagan), virologists (Delaha and Stewart), professor and director of Livestock Sanitary Service (Brueckner).

RESULTS

Upon electron microscopic examination of the concentrated suspension of the chorioallantoic membrane inoculated with the suspension of normal chorioallantoic membrane, no virus-like particles could be seen. These controls were screened carefully. Upon examination of the concentrated specimen from the infected papilloma virus chorioallantoic membranes, uniform virus-like particles were demonstrated as shown in figure 1. These bodies measure 55 mμ by direct measurement and are spherical in shape. These bodies resemble the papilloma virus bodies described by Melnick *et al.*[3]

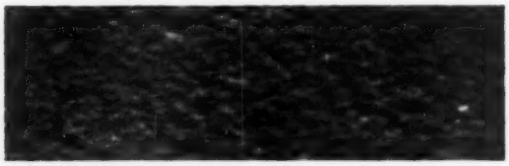

Fig. 2—Electron micrographs of concentrated normal chicken embryo chorioallantoic membranes. x 50,000.

SUMMARY

Studies by electron microscopy of chorioallantoic membranes of chicken embryos infected with Bivins papilloma virus show the virus to be spherical in shape with a diameter of 55 mμ. These bodies could not be demonstrated in concentrated suspensions of normal chorioallantoic membranes subjected to the same procedure of concentration and examination. The egg-adapted papilloma virus bodies resemble the papilloma virus described by Melnick *et al.*[3] in a previous paper.

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